Observations on:

THE EFFECTS OF CORTICO-STEROIDS AND OF GLYCYRRHETINIC ACID ON SALT AND WATER METABOLISM.

BY

E.E. GALAL,

M.B., B.Ch. (Cairo)

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Introduction

The role played by the adrenal cortex in regulating water and electrolyte metabolism has been recently the subject of an increasing number of investigations. One of the main obstacles in this field is the lack of an accurate and easily reproducible method of assaying mineralo-cortical activity. It is becoming almost a tradition for all workers in this field before proceeding with their investigations, to develop their own method of assay.

While some of the methods available have a reasonable degree of accuracy, in the hands of different workers they do not always prove to have the same degree of sensitivity and reproducibility as they had in the hands of their authors.

The mineralo-cortical activity of the cortical hormones is a complex function affecting different renal as well as extra-renal factors. The different methods of assay developed, of necessity, measure the effect of the cortical steroids only on some of the factors involved.

Much/

Much knowledge can be gained by the comparative study of the results of these different methods of assay. The interpretation, however, is rendered difficult by the fact that the response measured vary from one method to the other.

The understanding of the over-all role played by the adrenal cortex in regulating mineral metabolism, would be helped by the introduction of a simple and reproducible method of assay which discriminates between mineralo-active and non-active corticoids.

Such a method is included in this paper in the hope that it may prove to be easily reproducible in other laboratories.

Review of Methods of Assaying Mineralo-cortical Activity.

These methods can be classified as follows:

A. Chemical.

B. Biological.

I. Resistance to intoxication or poisoning.

(a) Water intoxication.

(b) Potassium poisoning.

II. Changes in urinary excretions of sodium by normal animals.

III. Changes in water or electrolyte balance in adrenalectomised animals.

1. Blood electrolytes.

2. Urinary excretion of:

(a) Sodium.

1. Radioactive isotopes.

2. Ordinary sodium salts.

(b) Potassium.

(c) Water.

A. Chemical Method/

A. Chemical Methods.

A non-biological method for estimating DOC and DOCA has been developed by Gordon and Pelly (1951). They utilised the principle developed by Heard et al (1946) that the reducing power of lipid soluble extracts prepared from urine provides a measure of the corticosteroid content of such extracts.

One ml. of 50 per cent methyl alcohol containing the steroid (DOCA) is heated to 100°C for three minutes with 2.0 ml. of Hagedorn-Jensen alkaline ferricyanide reagent. After rapid cooling the solution is made up to 6.0 ml. with distilled water. 1.0 ml. 10 per cent ammonium molibdate and 5.0 ml. glacial acetic acid are added. The colour produced is stable for more than one hour and can be estimated on a Spekker photoelectric absorptiometer using an Ilford violet filter. A blank experiment serves as control.

50-150 µg. of DOC can be estimated. The authors claim that reproducible standard graphs can be obtained over a range up to 200 µg. with an accuracy of ± 5%. No appreciable difference was detected/

detected between DOC and DOCA.

Promising results are reported by the authors to have been obtained with lipid extract of urine.

DOC is not thought now to form a part of the naturally secreted steroids. Any non-biological method of assay for mineralo-corticoids has to await the availability of the natural salt hormone before its usefulness in biological research can be tested.

No information about the limits of sensitivity of this method has been published and more information about the specificity of the method also is needed.

The lines along which this method has been developed may ultimately supply the research worker with a method of estimating mineralo-corticoids which is free from the uncertainties and hazards of the biological methods.

A method which appears to be developed along these lines is that of Pfeffer et al (1952). They reported on a method of chemical separation and estimation of 11-oxycorticoids and 11-desoxycorticoids. The separation depended on difference in degree of solubility in watery phases. When the steroids were allowed/

allowed to get distributed between a watery phase and a petroleum ether phase, 90% of desoxycorticosterone stayed in the petroleum ether while 90% of the cortisone passed to the watery phase. After separation the corticoids were extracted in chloroform. The chloroform solution was divided into two equal halves. After getting rid of the chloroform by evaporation under low pressure one of the two halves was heated at 100°C. for one hour in the presence of $\frac{N}{10}$ sodium hydroxide. This portion was then acidifed and dried to act as a blank in photometric estimation. The dry residues were dissolved in glacial acetic acid and with 0.5 ml. of a molybdenum phosphate reagent were heated on a boiling water bath for one hour then they were made up to 5.0 ml. with glacial acetic acid. The intensity of the colour was measured on a Pulfaich-Stufen photometer using an S_{72} filter. When 100 µg. of mineralo corticoids were added to the urine before extraction 85% were recovered in the mineralo-corticoid extract and 10% in the glucocorticoid extract. This method has a reasonable degree of accuracy. It seems however, to require large volumes of the test material which may be at times, a serious disadvantage.

B. Biological Methods.

I. Resistance to intoxication.

(a) Water intoxication. Eversole et al (1942) used the ability of cortical hormones to protect against water intoxication in developing a method of assay.

In adrenalectomised rats cortical hormones gave a certain degree of protection against water intoxication. Individual variations however, impeded the use of the assay as a quantitative method.

A comparative study representing mean results of many experiments showed that if DOCA potency is taken as unit, cortisone was three times as potent.

(b) Protection against potassium poisoning. Truszkowski and Duszynska (1940) investigated the protecting power of cortical hormones against potassium poisoning.

In intact male mice of 8.5 - 12.5 g. weight given an intraperitoneal injection of 2.5 ml. of 5 per cent potassium chloride death occurred within 5-10 minutes. Animals surviving more than 15 minutes showed no untoward after effects.

The/

The authors found that lowering of LD50 following adrenalectomy was a specific effect of adrenal deficiency in these animals. They proposed a method of assay using non-adrenalectomised mice of 9-11 g. weight.

A logarithmic dose effect curve was prepared for doses from 0.125 mg. DOCA to 1.0 mg. DOCA; the effect was taken as percentage mortality.

A group of thirty or more mice was used for the test substance. This group received the same load as the standard groups used for preparing the dose effect curve (7.5 mg. potassium chloride for 10 g. body weight intraperitoneally). A suitable dose of the test material varied between 0.3 - 0.5 mg. DOCA equivalent. The percentage mortality for the test material is calculated and the potency of this material can be estimated from the dose effect curve.

The authors claim these advantages for their method.

1. Cheap test animals were used.
2. Small amounts of the test materials required.
3. No adrenalectomy was performed.
4. A rapid method of assay giving its final result in 24 hours.

They/

They report a linear relationship between percentage survival and log dose of DOCA below 0.5mg.

The method however, does not have a great degree of sensitivity. No information about the degree of accuracy obtained nor about the response to other cortical steroids has been published. The use of non-adrenalectomised animals in most of this kind of assay diminishes the sensitivity of the method.

Feil and Dorfman (1945) developed Truszkowski and Duszynska's method (1940). They used adrenalectomised rats and claimed that these were a more sensitive preparation than adrenalectomised mice on the basis of body weight.

An interval of two days was allowed to elapse after adrenalectomy. They found that a rather delicate balance existed between a number of factors.

1. The time of administration of the potassium load and the administration of the cortical steroid.

2. The concentration of the potassium load.

3. The quantity of the active steroids administered.

The/

The optimum time between the administration of the potassium load and the injection of the active steroids was between 4 and 6 hours. The correct dose of the potassium load had to be found by trial and error before each experiment was run.

With their method, the authors obtained significant protection against potassium intoxication by:

a) 0.15 ml. of cortical extract;

b) 0.75 mg. of DOCA;

c) extracts equivalent to one litre of human urine.

This method, in addition to requiring a run of preliminary experiments to estimate the optimum potassium load, still has a low degree of sensitivity. No information was published about the degree of accuracy.

II. Non-Adrenalectomised Animals./

II. Non-Adrenalectomised Animals.

Urinary sodium.

Harrop et al (1937) proposed the use of normal dogs for assaying cortical extracts. The dogs were maintained on constant quantities of beef, sodium chloride and water. Urine was collected over 24 hours. Following subcutaneous injections of 2000 dog units of the extract reduction in urinary sodium excretion was most pronounced between the fourth and sixth hours, but was still detectable in a 24 hour collection.

Sodium excretion was inhibited. The concentration of sodium in urine as well as the quantity of sodium in the whole urine collection being diminished. Potassium excretion on the other hand was increased.

A significant decrease in sodium excretion was achieved by injecting 300-500 dog units to the test animal twice daily. Changes in potassium excretion were not as constant as those of sodium.

The authors proposed that a reduction in urinary sodium output of more than 5 milli-equivalent is significant as this quantity represented the maximal variations/

variations in their control experiments.

By obtaining the minimal amount of cortical extracts necessary to produce in a 24 hour collection, a 15-20 per cent reduction, this method can be used for assaying purposes.

As the method stands its limits of sensitivity does not allow for its use in the current research on mineralo-corticoids. No results were published demonstrating its accuracy or reproducibility.

The authors were aware that adrenalectomised animals may prove to be a more sensitive preparation for such purposes.

Hartman et al (1941) followed the same procedure as Harrop et al (1937). They used non-adrenalectomised female dogs. The animal weight was kept constant by regulating its feeding.

After a water load was administered, a six hours collection of urine was carried out. Test materials were injected subcutaneously dissolved in peanut oil or 10 per cent alcoholic solution.

The dogs' kidneys had to be in a healthy condition.

Results showed that individual responses varied over/

over a wide range; in one dog no response could be elicited. In hot weather accentuated variations prevented the use of the method. The results were considered reliable only if no decrease in potassium excretion was observed and no undue waterretention that could not be explained by the degree of sodium retention.

No analysis of the sensitivity, reproducibility or accuracy of this method were reported.

III. Adrenalectomised Animals./

III. Adrenalectomised Animals.

1. Blood electrolytes.

Lockett (1951) using female adrenalectomised dogs developed a method of assaying mineralo-corticoid activity. She used the ability of a cortical extract to stabilise the plasma chloride concentration in a dog in a state of mild insufficiency.

Two dogs are required for every assay. Constant environmental temperature and strict routine had to be observed. A diet high in potassium content and low in sodium was claimed to increase the sensitivity of the preparation. Standardisation of feeding necessitated persuasive hand feeding.

Animals which were not in a state of deficiency responded rather slowly to change in doses. In such a case, the time required to perform an assay is between 30-40 days.

In cases of advanced suprarenal deficiency symptoms of severe insufficiency were liable to interrupt the tests following a small reduction in the dose of extract used. The animals required a longer rest period between two successive experiments.

The two dogs employed were maintained at a mild degree/

degree of insufficiency. By reducing the dose of standard extract given this state was allowed to develop in dog No.2.

Dog No.1 was used to arrive at a rough estimation of the potency of the test extract. Doses of the test extract were administered in quantities which caused rapidly advancing deficiency. The curves for variations in plasma chlorides, non-protein nitrogen and haematocrit values for the standard extract had to be determined beforehand on this dog.

In four days, by comparing the slopes for the unknown and standard extracts, a rough estimation of the relative potency of the test extract could be arrived at.

Dog No.2 was then used for the second step. A dose of the test extract corresponding to the requirements of a period of 6-8 hours was administered and the changes in the plasma chloride, non-protein nitrogen and haematocrit values were determined. The dose of the standard extract just more effective and that just less effective than the standard could then be estimated.

This second step was then repeated again on dog No.1./

dog No.1. By diminishing the maintenance dose of the standard extract a state of slowly advancing deficiency was firstly established in this dog.

A satisfactory degree of accuracy was reported by the author (limits of accuracy ± 6 per cent). The quantities needed of the test material for a complete assay however, prevent the use of this method in the current research work on the adrenal cortex. In addition, the time required to complete the assay can hardly allow for its use as a routine method of assay in such a research. After determining the dose effect curves for the standard extracts a period of 24 days is still needed to finish the assay.

The method was intended for assaying large quantities of commercial extract and undoubtedly has its possibilities in this field.

2. Urinary Sodium.

Ordinary sodium salts. Deming and Luetscher, (1950) used adrenalectomised male rats to assay mineralo-corticoids. Twenty-four hours before the test, the rats were deprived of the saline drink on/

on which they have been maintained since adrenalectomy. A water load was then given and the test material administered subcutaneously in an ethanol solution. Urine was collected over 5 hours. On the previous day each rat supplied its own control values.

The rats had to be tested beforehand and any rat with a control value of less than 30 µ. equivalent had to be disregarded, also rats which were unfit or their urine secretion below 0.3 c.c.

They reported group variation from day to day over a wide range.

No dose effect curve is supplied and no reference to accuracy.

Simpson and Tait (1952) criticised this method for the above mentioned reasons.

Kagawa et al (1952), developed a method using male albino rats 150-155 g, and a saline load subcutaneously. The steroid was given in corn oil.
In an attempt to get a complete collection of urine, the urethra was ligated at the outset and the bladder dissected out and washed at the end of the collection and the volume of the urine was measured. Proteins and/

and phosphates had to be precipitated before estimating sodium colorimetrically. Significant sodium retention was achieved by doses of 2.0 µg. DOCA or above $P < 0.01$ significance difference of sodium excretion between doses 2 and 4 µg. DOCA was achieved ($P < 0.05$).

A dose effect curve for doses between 1.0 to 12.0 µg. DOCA was reported with a satisfactory degree of precision ($\lambda = 0.256$). The animals were used 24 hours after adrenalectomy and collection was complete over two hours. Five hours after giving the sodium load the collection was started and the first half dose was given. The other half dose of the DOCA was given four hours after the load. Distilled water ad. lib. was allowed during collection time.

Compared to other available methods this one seems to cover a reasonable degree of accuracy. The procedure however, is laborious and no study of the cortical steroids was reported.

Spencer (1950) using adult adrenalectomised male mice reported a method of assaying minero-cortical activity by utilising the sodium retaining effect of/

of DOCA.

The animals had to be tested preliminarily to select those excreting between 70-130 per cent of the sodium load. Twelve animals were required for each assay, by dividing them into three groups, one acting as control, the other as standard and the last as test. The groups were crossed over on three successive days, each animal supplying its own control, test and standard values.

Spencer reported a linear relation between log dose of DOCA and percentage sodium retention over a range 0.5 - 4.0 µg.

A mean difference of sodium retention of 15 per cent is significant at a level of probability (P) 0.02.

In my hands, this method did not yield the same degree of accuracy. The response of the same mouse varied from a day-to-day over a wide range. Some mice died or had to be discarded because of exhaustion from starvation and handling. Adult mice did not seem the ideal animals for this kind of experiment. In a number of strains a high proportion were able to/

to survive adrenalectomy and deprivation of sodium chloride drink for more than 8 days. Other strains proved to be difficult to adrenalectomise completely.

Simpson and Tait (1952) found this method very time consuming as it required a preliminary test for the selection of suitable experimental animals followed by a cross over test of 6 hours on three successive days.

Radioactive sodium and potassium.

Dorfman et al (1947) reported a new method of assay using adrenalectomised male albino rats and radiosodium (Na_{24} and Na_{22}) in a sodium chloride solution as a carrier.

They found that urinary sodium excretion was increased in adrenalectomised rats. The rate of increase seemed to reach its maximum around the fifth day after adrenalectomy.

In non-adrenalectomised rats given a load of hypertonic solution of sodium chloride containing 0.35 mg. sodium chloride per g. body weight in 1 ml. Sodium retention caused by 1000-2000 µg. DOC was low. When/

When however, a smaller load (0.035 mg. sodium chloride per g. body weight in 2.0 c.c.) and of lower tonicity was used the ratio of sodium retention was much improved.

The rate of sodium output in the urine of adrenalectomised animals varied over a wide range. The rate of sodium excretion of different batches of animals seemed to fluctuate from one experiment to another over a wide range.

No. of rats	Mean wt.	DOC	Mean Na excretion %	Mean control Na excretion % / Mean standard Na excretion %
8	139g.		6.69	
6	141g.	25µg.	1.31	5.1
9	145g.		2.04	
11	138g.	1.95µg	1.35	1.51

Thus the need for relating the sodium excretion of the standard groups to that of the control of the same group was demonstrated.

In their method significant retention was caused by a minimal dose of 0.98 µg. of DOC and 10 µg. DOCA.

The intensity of sodium retention effect from dose to dose was observed only with doses below 7.82 µg. DOC per rat.

The

The report was not accompanied by a dose-effect curve. In a later report, (Dorfman, 1951) a study of the effect of other steroids was published. Hydrocortisone 25 µg, testosterone 2,000 µg. and oestradiol 2,000 µg. caused no significant sodium retention.

This method suffers from the same handicap as other methods employing radioactive isotopes. Deming and Luetscher (1950) found this method to entail certain technical difficulties in procuring and measuring the variable specific activity of these isotopes.

Simpson and Tait (1952) extending the work carried out by Dorfman (1947-49) developed a method for assaying mineralo-cortical activity by measuring the $\frac{Na^{24}}{K^{42}}$ ratio in the urine of adrenalectomised rats. They argued that only radioactive compounds can allow for the use of very small loads and thus a true balance experiment can be carried out under physiological conditions. The sodium potassium ratio offered certain advantages.

a) Errors in volume measurements of the load solution were neutralised.

b) In the final preparation of the urinary samples sputtering and decay corrections could be neglected/

neglected.

c) The completeness of urine collection which is a vital step in all the methods depending on the estimation of one electrolyte only, was not of such importance in their method.

Male Wister rats, 30-40 g. in weight were employed on the fifth day after adrenalectomy. One hour before the load, the steroid was injected subcutaneously in 0.1 ml. 20 per cent alcohol. The load of labelled Na^{24} and K^{42} was prepared to contain 27 µg. sodium and 381 µg. potassium in 0.5 ml. water and adjusted to pH 7.5. Total radioactivity was approximately 10 µg. curies on the day of the assay. Urine collection was for a period of two hours at the end of which suprapubic pressure and ether were utilised to empty the bladder. The $\frac{Na^{24}}{K^{42}}$ ratio was then determined in the urine as well as in the load by using a special physical absorption technique.

In the case of DOCA a significant negative regression of urinary $\frac{Na^{24}}{K^{42}}$ with dose was obtained ($P < 0.001$). The linearity of the relation between this ratio and the logarithm of the dose was not in doubt. There was however, a significant difference between the different results obtained on different days/

days. The index of precision for two sets of results was 0.267 and 0.295.

The same type of effects were obtained with cortical extracts. The minimal effective dose per rat was that equivalent to 0.0049 ml. of a commercial extract.

The mean index of precision being 0.30, the authors advised the use of 24 rats for the test as well as for the standard doses.

The differences between different blocks of results, necessitated the simultaneous use of standard and unknown in each assay.

To facilitate the choice of the appropriate doses for the quantitative assay, a standard curve can be used to determine the approximate potency of the test material.

In such a case, expression of the ratios as percentages of the control values of non-treated animals of the same set resulted in the disappearance of the differences between blocks.

For comparing different steroids four or six-point assays and 24 rats per compound were used.

Desoxycorticosterone, desoxycorticosterone acetate, corticosterone, 11-desoxy-17-hydroxycorticosterone acetate/

acetate, 17,hydroxy-corticosterone, 11-dehydrocortico-sterone acetate and 11-dehydro-17-hydroxycorticosterone acetate were tested and found to be active in descending order of potency. No compound was found which raised the $\frac{Na^{24}}{K^{42}}$ ratio. With a larger sodium load and a larger collection period however, cortisone failed to cause lowering of the sodium potassium ratio.

In their comparative study of different steroids the authors found that there was qualitative and quantitative correspondence with the results of the survival time of Cartland and Kuizenga as well as the Everse-de Fremery test. The authors point out the advantage of being able to include steroids of short term action because of their collection time of two hours.

This method offers a high degree of sensitivity and accuracy.

The technical difficulties involved however, are great. In addition to the preparation and use of radioactive isotopes, a special physical absorption technique is needed to estimate the sodium potassium ratio. Twenty-four animals are used per group and a 4-6 point assay including a control group is needed. The method does not differentiate qualitatively between mineralo-corticoids and glycocorticoids.

Marcus/

Marcus et al (1952) developed a method of assay using radioactive sodium. Male rats of 150-175 g. weight were used. The sodium load (5.0 ml. saline) was administered intraperitoneally and the test material subcutaneously in olive oil. Urine was collected from every pair of rats together over a period of 4 hours. On studying water, potassium and sodium excretion the latter alone was found to furnish the most reliable indication of mineralo-cortical activity. From 52 pairs of rats the data obtained from sodium excretion gave λ value as 0.351. The range of sensitivity of the assay was 6.0 - 60.0µg. DOCA.

Corticosterone caused no sodium retention in all doses used.

Cortisone and corticosterone were the most effective diuretic steroids at high doses for water as well as sodium.

Below 10 µg. doses all the steroids tested, except corticosterone, caused some sodium retention; above that dose all cortical steroids tested enhanced sodium excretion apart from DOCA.

This method has the advantages of a reasonable degree/

degree of accuracy and of giving an idea of the degree of diuresis accompanying electrolyte changes. Its minimal effective dose of 6.0 µg. DOCA is rather high. The method has the same disadvantages as the other methods using radioactive salts.

Singer et al (1953) using radioactive sodium and adult male hooded rats reported a method of assay. The sodium load contained 3.5 mg. of sodium chloride with tracer quantities of Na^{24}. Precautions were taken to ensure complete collection of urine by ligating the urethra and removing urine with a syringe. The mean excretion of the control group was considered as 100 per cent excretion and values for test or standard expressed as percentages of this mean. Test substances were given in 0.1 ml. absolute alcohol, 0.25 ml. corn oil or 0.25 ml. 25 per cent alcohol. Urine was collected over 6 hours.

The results for DOCA pooled from three experiments gave significant sodium retention at a dose of 2.0µg. ($P < 0.01$).

The results for cortisone were not constant. In the majority of cases, there was a tendency to enhance/

enhance sodium excretion.

As in the experiments of Kagawa et al (1952) and Simpson and Tait (1952) the results were expressed as percentages of the results obtained with control animals on the same day.

The authors reported that sodium excretion in adrenalectomised animals was very variable, even under similar conditions.

Statistical analysis of the data of this method is not complete and more information about other steroids is needed. This method again entails the use of radioactive isotopes.

3. Urinary Potassium

Dorfman (1949) using radioactive potassium and adrenalectomised albino rats developed a method of assay, depending on the increase in potassium excretion by adrenal deficient DOC treated animals.

The test is carried out 24 hours after adrenalectomy. No saline drink was given. The potassium load contained 20 - 280 µg. of the K isotope per g. body weight in 2.0 ml. solution.

Urine is collected over 6 hours and potassium excretion is expressed as per cent of the load.

With/

With the higher potassium loads employed the authors reported a significant increase in urinary potassium with 10.0 µg. DOC given subcutaneously in 0.25 ml. corn oil.

No attempt at defining the method as a quantitative assay is reported. The sensitivity of the method does not seem to compare favourably with those methods measuring sodium excretion. The same technical difficulties encountered with radioactive sodium would be encountered with the potassium isotopes used here with half a life of 12.8 hours.

4. Water Diuresis

Petranyi (1941) used the diuretic effect of cortical hormones in hydrated adrenalectomised animals to develop a method of assay.

Water loads of 5 per cent of the body weight were given to adrenalectomised rats by stomach tube. In the control untreated rats, urine volume did not exceed 25 per cent of the load while after treatment with cortical hormones diuresis ensued. The amount of cortical hormone just sufficient to give a urine volume 50 per cent of the water load was taken as one rat diuresis unit. 2 mg. of DOCA was equal to a unit.

Methods

A. A qualitative biological method for detecting mineralo-cortical activity.

All the known methods for assaying mineralo-cortical activity entail both the use of a large number of animals and complicated procedures. These methods can only be used in a limited range of doses. Precious time and material can be saved if a rough knowledge of the potency of the test material is known beforehand, thus avoiding the possibility of using doses beyond the range of sensitivity of the method of assay.

A method for the identification and rough estimation of mineralo-cortical activity is proposed here. The method has the advantage of requiring a small number of animals and a reasonably simple procedure. Several test materials can thus be estimated roughly in one day and only small amounts of them are required.

Method: Male albino rats 150 - 200 g. were adrenalectomised under ether anaesthesia. The animals were fed on rat cubes and supplied with a drinking solution containing 1 per cent sodium chloride/

chloride and 5 per cent glucose. On the third day cubes were withdrawn at 5 p.m. and drinking solution replaced by a 5 per cent glucose solution. The animals were kept in a quiet room at a constant temperature of about 22°C.

At 11 a.m. next morning the animals were weighed and given 5 ml. per 100 g. body weight of a solution of 14 per cent redistilled ethyl alcohol in 5 per cent glucose solution. The solution was introduced by stomach tube. The rat's mouth was pushed gently against a glass tube covered by a well fitting rubber tubing. When the rat opened its mouth its head was manipulated to introduce the tip of the tube inside its mouth without pushing in its tongue. A thin rubber catheter connected to a glass syringe containing the required solution was pushed through the glass tube down the rat's oesophagus until a mark indicating the required length to reach the stomach. The solution was then injected slowly and the catheter withdrawn. The rats were then kept in a quiet and darkened place at a constant temperature. They started to get drowsy in about 10 minutes and were generally under light anaesthesia in 30 minutes.

At/

Figure 1

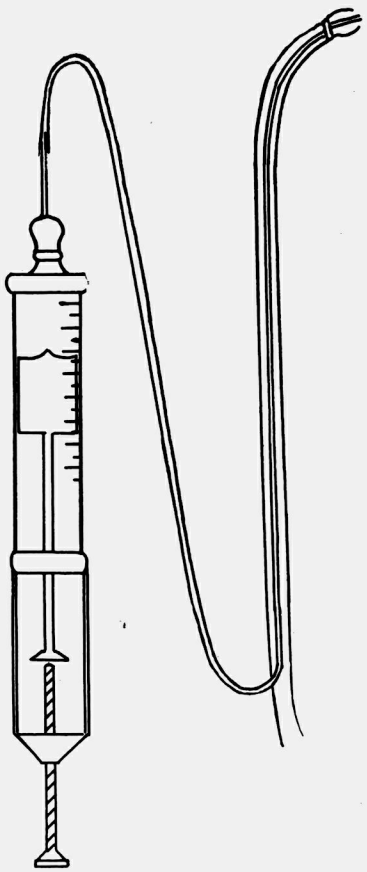


FIG. 1

APPARATUS FOR WASHING THE URINARY BLADDER OF THE RAT.

At 12 noon, 4.0 ml. of 0.9 per cent sodium chloride solution was injected subcutaneously to each rat. A long fine hypodermic needle was used. The needle, introduced near the tail was pushed under the skin to the shoulder region and the solution evenly distributed. Ten minutes later, 0.1 ml. of watery solution of "Percorten" containing a known amount of the active substance was injected to the test animals while the control animals got the corresponding amount of water only. The injection was given intra-peritoneally.

Thirty minutes later a catheter was introduced suprapubically mostly without the need of any anaesthetic. In some cases, however, the anaesthesia being light, a few whiffs of ether were needed.

The catheter used was a double walled polythene one. The outer tube had an external diameter of 3.5 mm.; the inner tube of 1.5 mm. (Fig.I); the thinner tube is connected to a blunt needle fitted in a 2 ml. syringe fixed in a micro-feeding-screw.

The penis of the rat was secured in a firm ligature to prevent any escape of urine.

A suprapubic longitudinal incision 1.5 cm. long was/

was made in the middle line. The bladder secured and without any pulling, and using a pair of sharp fine scissors the catheter was introduced through a small incision in the apex of the bladder and tied with a fine silk ligature. Kinking the bladder or damaging its wall were completely avoided. A small amount of distilled water was then injected through the inner tube to test the patency of the catheter, minimum pressure being used.

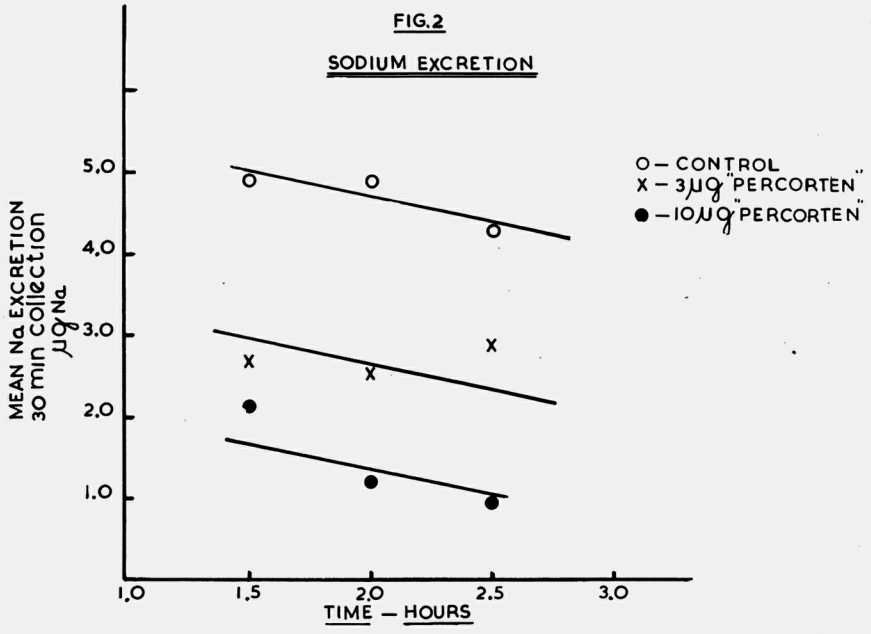
The rats were then put on a specially heated box whose temperature can be adjusted by switching on or off electric bulbs.

A thin thermometer was introduced per rectum and fixed to a piece of plasticine to prevent it falling out.

The rats temperature was kept as near to 37°C. as possible throughout the experiment, and noises were avoided throughout the experiment. The time of injecting the sodium chloride load solution was considered zero hour.

At 1.10 each bladder was washed out by injecting 2.0 ml. of distilled water through the inner tube the solution coming out being discarded; after that, a graduated centrifuge tube was used to collect any fluid dripping from the catheter. Every 30 minutes the bladder was washed again by injecting/

Figure 2.



Effect of "Percorten" on Sodium Excretion in Adrenalectomised Rats.

(Table I)

injecting 2.0 ml. of distilled water through the inner tube of the catheter, the injection being done by the micro-syringe, so that a steady slow flow of the water was maintained. The washings were collected in the same centrifuge tube which was then replaced by a new one. Thus all the urine secreted every 30 minutes was collected in one tube. The collection was continued for one hour and a half, at least.

The fluid in the centrifuge tubes was then made up to 10 ml. and the quantity of sodium in it estimated by the flame photometer.

Results.

As shown in the accompanying table, the amount of sodium excreted over 50 minutes, beginning from one hour and a half since the injection of the sodium load, varies with the amount of "Percorten" administered.

In these experiments, like others under similar conditions, sodium excretion by control animals showed marked variation from one animal to another. This individual variation tends to diminish the reliability of the test. (Table I, Fig. 2).

Any/

TABLE I.

Effect of "Percorten" on Sodium Excretion.

Expt No.	Time of collection.	RAT I		RAT II		RAT III	
	Mins.	Dose "Percorten"	Na excretion in 30 mins. µg. Na per min.	Dose "Percorten" µg.	Na excretion in 30 mins. µg. Na per min.	Dose "Percorten" µg.	Na Excretion in 30 mins. µg. Na per min.
1	90	0.0	3.51			10.0	2.21
	120		2.89				1.70
	150		3.50				1.50
2	90	0.0	5.00	3.0	1.70	10.0	0.56
	120		6.60		1.90		0.23
	150		3.70		3.10		0.20
3	90	0.0	7.80			10.0	2.90
	120		6.10				1.03
	150		8.30				0.90
4/							

TABLE I Contd:

Expt. No.	Time of collection Mins.	RAT I		RAT II		RAT III	
		Dose Percorten	Na excretion in 30 mins. µg. Na per min.	Dose Percorten ug.	Na excretion in 30 mins. µg. Na per min.	Dose Percorten µg.	Na excretion in 30 mins. µg. Na per min.
4	90	0.0	3.13	3.0	3.93	10.0	2.27
	120		3.63		2.26		1.13
	150		2.36		2.90		1.13
5	90			3.0	2.36	10.0	2.63
	120				3.33		1.93
	150				2.53		0.90
Mean	90		4.89		2.66		2.12
	120		4.81		2.50		1.20
	150		4.24		2.84		0.93

Any damage to the bladder while introducing the catheter tended to produce haematurea or partial or complete anurea, and in such cases the animal had to be discarded.

In some animals difficulty in breathing was caused by mucous secretion in one or the other of the respiratory passages. Attempt to avoid this by introducing a tracheal cannula resulted in a degree of surgical shock which interfered with urine excretion and the results were not reliable. Animals developing difficulty in breathing were therefore discarded.

Constant temperature and complete protection from interference were found to be important. The alcohol anaesthesia was light enough to allow responses to very loud noises or a sudden exposure to a strong source of light.

The degree of anaesthesia also was found to play an important role. Animals with light enough anaesthesia to permit them to perform any degree of spontaneous movements as well as animals with too deep a degree of anaesthesia had to be discarded.

The amount of alcohol necessary to produce the required degree of anaesthesia under the conditions of/

of the experiment had to be investigated for every new batch of rats received.

Under the above conditions it was decided that to use this method as a quantitative method of assay would entail using four groups of animals each group consisting of several animals. It is difficult for an operator to deal with more than 8 animals at a time thus the amount of labour and time necessary would not be justifiable by the degree of accuracy expected. On the other hand, this method can be of some use as a preliminary attempt at a qualitative estimation of mineralo-cortical activity. In experienced hands one group of 8 rats can be handled to give a rough idea of the mineralo-cortical activity of one sample. The final result can be obtained in a few hours. Used in conjunction with an accurate method for quantitative estimation, this method can be of help in selecting the optimum dilution of the test material.

B. A Quantitative Method for Assaying Mineralo-cortical Activity.

In an attempt to use Spencer's (1950) method for assaying DOCA, I failed to achieve the degree of accuracy obtained by the author. The reproducibility of mineralo-cortical activity assays seems always to be subject to certain difficulties. It has been the usual procedure up till now for almost every investigator to introduce his own method or at least introduce some modifications to one of the already published methods.

Unfortunately, I was forced to follow the same trend. It was thought advisable to record some of the investigations carried out during the process.

The ultimate aim of a successful method of assaying mineralo-cortical activity relying on urinary salt excretion seems to be to achieve a state of affairs where adrenalectomised animals in a moderate degree of cortical insufficiency are able to excrete a consistently high percentage of a given load of sodium.

The variability of sodium excretion over a wide range in adrenalectomised animals has been a persistant finding by many workers. The plasma sodium

level follows the same variable pattern. These variations occur between individual animals as well as between groups of animals. Harrop and Thom (1937), Deming and Luetscher (1950), Spencer (1950), Simpson and Tait (1952), Singer and Venning (1953), Bronwell et al (1950) and Morel (1951) observed the inconsistency of sodium excretion by adrenalectomised animals.

The usual procedure, followed in most previously discussed methods of assay, of depriving the adrenalectomised test animals of sodium for some time before the experiment and then giving them a known load of sodium, does not seem to correct this variability.

To overcome this handicap, several procedures have been proposed:-

The use of radioactive Na, K, or Na + K.

Selecting animals with a high urinary output of sodium and water.

Using the same animal to supply its own control values as well as the test and standard values.

Using a large number of animals for each dose of test and standards, as well as a large control group on the day of the assay.

Different amounts of sodium at different osmotic pressures.

Different/

Different times for the collection of urine and the duration of the collection.

Different species of animal etc.

The use of radioactive isotopes involves certain technical difficulties for procuring, preparing, as well as estimating, these isotopes. Na^{24} has half-a-life of 14.8 hours which does not allow for its convenient use except in especially favoured laboratories. Na^{22} with a half-life of three years is difficult to procure. The different factors involved in the other procedures are discussed in the following paragraphs.

A study of some of the factors affecting the test.

(a) Using the same animal to supply its own control, test and standard values:

(1) Mice: (no pre-treatment with corticoids). Using an adrenalectomised animal on successive days including periods of starvation from food as well as sodium chloride with the necessary handling for injections and emptying of the bladder etc. did not prove to be the ideal procedure in my hands. When a day of rest was allowed in between every two tests the length of time required to finish the assay became/

became too long to allow any degree of stability in the rate of sodium excretion. Sodium excretion in adrenalectomised rats was shown by Dorfman et al (1947), to reach its maximum around the 5th day and then to decline gradually. Individual variations from day to day after adrenalectomy are illustrated by the following experiment.

Adrenalectomised mice of average weight of 35g. were given 10% sodium chloride and 5% glucose to drink at first. The sodium chloride was then removed and they were given only 10% glucose to drink for a period of 12 hours before the assay. A solution of sodium chloride containing 6.348 mg. of sodium was injected subcutaneously and the animals removed to the metabolic beakers (for technique see later), for a urine collection of 9 hours. This was carried out once on the 3rd day after adrenalectomy and another time on the 7th day. In between these experiments the mice were again maintained on the saline drink.

Table II shows the results for each collection from different mice on the 3rd and 7th day. The variations between the two collections from the same animal were very big. Animal No.1 for instance, excreted about 114% of the sodium load on the/

TABLE II.

Effect of Time since Adrenalectomy on Sodium Excretion.

(no pre-treatment with corticoids).

Mouse	Sodium excretion in 9 hours (mg. Na)	
No.	3rd day after adrenalectomy	7th day after adrenalectomy
1	7.29	0.50
2	8.86	2.03
3	6.05	3.35
4	0.51	2.59
5	6.84	5.65
6	0.34	5.75
7	7.79	7.67
8	5.76	6.86
9	4.30	1.95
10	3.15	7.25
Mean	5.09	4.36

Each mouse received 6.348 mg. Na subcutaneously.

TABLE III.

Effect of Time since Adrenalectomy on Sodium Excretion.

(Pre-treated with cortisone†)

Mouse No.	Sodium Excretion in 8 hours (mg. Na).							
	4th day	Mean	5th day	Mean	7th day	Mean	9th day	Mean
1	2.87						9.25	
2	2.89	2.90					9.50	8.78
3	2.93						7.58	
4	3.94				5.78			
5	2.70	3.76			2.67	5.33		
6	4.65				7.53			
7	6.26		4.78					
8	5.35	6.30	6.26	5.92				
9	7.29		6.71					

† Each mouse received 100 µg. cortisone subcutaneously on the 1st and 3rd day after adrenalectomy.

TABLE IV.

Effect of Time since Adrenalectomy on Sodium Excretion.

(Pre-treated with "Percorten"+)

Mouse No.	Sodium Excretion in 8 hours (mg. Na).							
	3rd day	Mean	5th day	Mean	7th day	Mean	9th day	Mean
1	5.08		7.96					
2	1.99	3.51	0.75	4.25				
3	3.47		4.04					
4	3.12				4.036			
5	4.11	2.72			8.20	5.89		
6	0.84				5.34			
7	1.23	1.97					0.83	3.21
8	2.70						5.59	

+ Each mouse received 100 µg. "Percorten" subcutaneously on the 2nd day after adrenalectomy.

the 3rd day while it excreted only about 8% of the sodium load on the 7th day. On the other hand, animal No.6 excreted only 5.3% on the 3rd day and 90.6% on the 7th day. It is interesting that while such individual variation from day to day covered such a wide range the mean sodium excretion for the whole group exhibited only a moderate variation (80.2% and 67.1% respectively).

(2) Mice: (pre-treated). Similar experiments were carried out on cortisone treated and "Percorten" (DOC glucosides), treated mice respectively. It was thought that such a treatment given to the adrenalectomised mouse before the exacting experiment of cross-over assay entailing starvation and repeated handling might furnish a more appropriate base line, by correcting the water and electrolyte shifts between the intracellular and extracellular compartments, and moderate the degree of insufficiency in the later part of the experiment. The same method as described above was used. In the case of cortisone treated animals each mouse received 100µg. cortisone on the 1st and 3rd days after adrenalectomy. The drug was given subcutaneously as a suspension in saline.

"Percorten"/

"Percorten" treated mice received 100 µg. in distilled water on the 2nd day after adrenalectomy.

The results are shown in Tables III and IV. They indicate that while a certain degree of stability may be achieved on the first two or three days after hormonal administration, this stability is not of a degree or duration to allow for animal selection and cross-over experiments.

The sodium excretion of animals tested on the 4th and 7th day after cortisone injection varied widely from one collection to the other. This is even more so with animals tested on the 4th and 9th day. Similar results were obtained with "Percorten" treated animals.

(b) Sodium excretion in relation to water diuresis in adrenalectomised mice.

It has been repeatedly observed in my experiments that animals failing to excrete reasonable amounts of sodium also failed to excrete water.

Stein and Wertheimer (1944) reported that adrenal medullectomy inhibited diuresis in hydrated animals. l-epinephrine returned the diuresis to normal.

Hays/

Hays and Mathieson (1945) found that epinephrine increased diuresis in adrenalectomised rats whether treated or untreated with cortical hormones. Gaunt et al (1945) confirmed their findings.

Drill and Bristol (1951) found that a saline load given to normal rats diminished the rate of urine flow compared with that from water loaded controls. Epinephrine or nor-epinephrine raised the rate to the control values. The doses used were 100 µg. per 100 g. body weight. The effect of this dose on chloride excretion was to increase the chloride concentration in the urine of water loaded animals. In saline loaded animals, however, the concentration was not raised and the total increase was proportional to the increase in the volume of the urine.

Horres et al (1950) reported that doses of 3-30 µg. epinephrine per 100 g. body weight had no diuretic effect.

An experiment was devised to test the possible effect on sodium excretion in adrenalectomised animals of adrenaline induced diuresis and whether such a procedure may correct the individual variations.

Method:/

Method: The same method as previously described was used. The mice had an average weight of 37.0 g. 30 µg. adrenaline B.P. was injected subcutaneously in 0.5 ml. distilled water to each animal and a collection of 6 hours was carried out. The group of mice used was originally divided into equal halves according to weight. One half acted as control and was not given adrenaline. On the 2nd day the two halves were crossed over the original control group receiving adrenaline, and vice versa. The test was carried out on the 4th and 6th days after adrenalectomy. Before the test and in between the two experiments, the animals were maintained as usual on 1% sodium chloride, 5% glucose solution for drink and on rat cubes.

Results: The results achieved show that no significant change in sodium excretion by the kidney was noticed (Table V). An appreciable increase in the volume collected was noticed in the adrenaline injected animals although the apparatus used did not allow for its measurement.

(c) Potassium/

TABLE V.

Effect of Adrenaline on Sodium Excretion in Adrenalectomised Mice.

	Control Mice Na excretion as mg. Na in 6 hrs.		Adrenaline Injected Na excretion as mg. Na in 6 hrs.
4th day	4.94	4th day	3.29
	2.96		1.18
	5.86		5.15
	0.67		0.77
	0.67		4.30
6th day	0.79	6th day	1.32
	0.75		2.99
	0.11		0.39
	1.09		3.40
	4.38		5.00
Total	28.22		27.79
Mean	2.82		2.78

Each mouse received 6.348 mg. Na subcutaneously.

(c) Potassium excretion.

Several workers have reported on the inconstancy of the urinary potassium response in adrenalectomised animals treated with cortical hormones; Roberts and Pitts (1952), Perera et al (1949), Sartorius (1951), Seldin et al (1951), Barnett et al (1949), Harrop et al (1937) and Marcus et al (1952).

Experiments (Table VI) have been carried out to study potassium excretion in adrenalectomised mice treated with "Percorten". (Fig.3).

The mice were bilaterally adrenalectomised under ether and kept on 1% sodium chloride, 5% glucose and rat cubes. A few days after adrenalectomy food was withdrawn from 5 p.m. and the salt drink was discontinued from 2 a.m. and replaced by a 10% glucose solution. The assay was continued as previously described, the mice being divided into equal groups according to weight, one acting as control, and the other groups being given doses of "Percorten". The groups were crossed over and the experiment repeated on two further days so that each mouse supplied its own control and test values.

In/

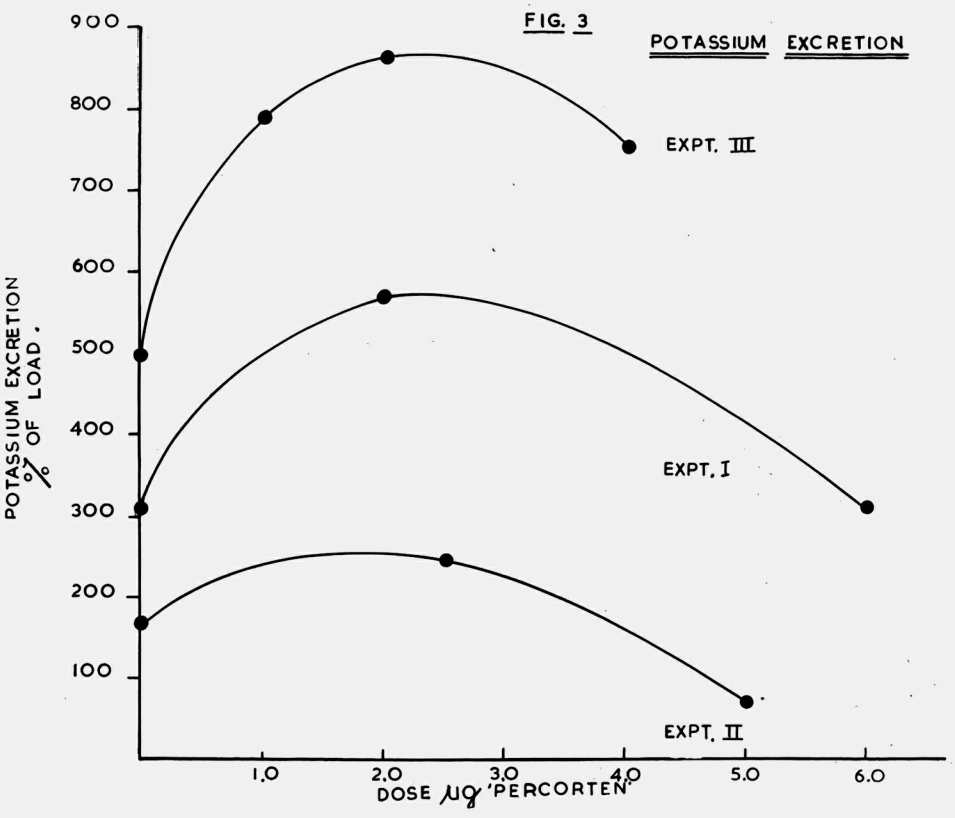
TABLE VI.

Effect of "Percorten" on Potassium Excretion.

Expt No.	No. of days since adrenalectomy.	Mouse No.	Load in 2.0 ml.	Duration of Collection	Potassium excretion[1] (% of load)			
					Control	"Percorten" 2µg.	"Percorten" 6µg.	
1	3, 4 and 6	1	0.34 mg. K + 6.348mg. Na	6 hrs.	729.0	1411.0	570.0	
		2			276.0	1264.0	173.0	
		3			238.0	261.0	188.0	
		4			353.0	424.0	276.0	
		5			217.0	132.0	247.0	
		6			305.0	459.0	471.0	
		7			018.0	044.0	256.0	
		Mean			305.0	570.0	312.0	
					Control	"Percorten" 2½µg.	"Percorten" 5µg.	
2[2]	5, 7 and 9	1	0.34 mg. K + 6.348mg. Na	6 hrs.	18.0	579.0	176.0	
		2			09.0	359.0	06.0	
		3			18.0	24.0	18.0	
		4			397.0	420.0	41.0	
		5			271.0	65.0	20.0	
		6			294.0	18.0	150.0	
		Mean			168.0	244.0	69.0	
					Control	"Percorten" 1.0µg	"Percorten" 2.0µg.	"Percorten" 4 µg.
3[3]	5, 7 and 9	1	0.17 mg. K + 3.174mg. Na	6 hrs.	253.0	588.0	1194.0	965.0
		2			465.0	947.0	1241.0	765.0
		3			318.0	865.0	488.0	871.0
		4			441.0	912.0	282.0	158.0
		5			35.0	576.0	782.0	671.0
		6			912.0	565.0	1529.0	1488.0
		7			824.0	918.0	1347.0	694.0
		8			700.0	965.0	29.0	400.0
		Mean			494.0	792.0	862.0	752.0

[1]The figures in each horizontal line represent results of experiments carried out on the same mouse.
[2]Mice in this group were given 100µg. on the 1st and 2nd day after adrenalectomy.
[3]Female mice used in this group were ovariectomised during the operation of adrenalectomy.

Figure 3.



Effect of "Percorten" on Potassium Excretion in Adrenalectomised Mice.

(TABLE VI)

In Experiment I the tests were carried out on the 3rd, 4th and 6th days after adrenalectomy. In Experiment II the tests were carried out on the 5th, 7th and 9th day after adrenalectomy. In Experiment III the tests were carried out on the 5th, 7th, 9th and 11th day after adrenalectomy.

In between tests the animals were maintained on 1% sodium chloride and 5% glucose solution and rat cubes.

Before beginning Experiment II, the mice were given 100.0 µg. cortisone subcutaneously on the 1st and 2nd day after adrenalectomy.

In Experiment III, female mice were used and they were ovariectomised during the operation of adrenalectomy.

Results: As is shown in Table VI and Figure 3 there was a tendency for the potassium excretion to increase with increasing doses of "Percorten" until a maximum excretion was achieved at a dose of 2.5-3.0 µg. After that, the curve started to decline. The amount of potassium excreted tended to exceed the small load injected even with the control group. No attempt at studying the behavious of higher loads of/

of potassium or of higher doses of "Percorten" was carried out. It was obvious however, that, under the conditions of these experiments potassium excretion could not be used for assaying purposes.

It is of interest that, inspite of the difference of time and procedure between the three reported experiments, each time the potassium excretion first rose and then fell when the dose of "Percorten' was increased.

(d) Other factors involved in developing a method of assay:

1. Animal species. It has been pointed out before that certain difficulties are involved in using mice for experiments on adrenal deficiency. Rats on the other hand, can be easily adrenalectomised and the adrenalectomy in such cases seems to be complete. Amongst a large number of experiments carried out involving more than a thousand young rats, only in very few instances did a rat survive saline drink withdrawal for more than 8 days.

2. Age and weight. From the point of view of supply, the younger the animals used the smaller the cost. Another factor is the degree of the sensitivity of the preparation. With bigger animals/

animals larger quantities of the active principle would be required and as the number of animals used in these assays is relatively high economy in test materials is essential.

In my experiments, rats below 45 g. weight at the beginning of the experiment did not withstand the fasting and necessary handling. A considerable number of these young animals had to be discarded because of exhaustion or death. The most suitable size was found to be between 60-80 g. body weight. The practical difficulties involved in securing a constant supply of large numbers of rats of this size did not allow for the use of animals of strictly standardised weight. Animals of 55-95 g. weight were sometimes included in the same test but the weights were evenly distributed among the different groups.

3. A mild degree of cortical insufficiency increases the sensitivity of the adrenalectomised animal to hormonal treatment. Dorfman et al (1947), found that the maximum sodium output in adrenalectomised animals kept on a saline drink was reached around the 5th day. Several workers have/

have chosen the 4th day after adrenalectomy to start their assays. It was found to be the most suitable day also for the present work.

4. Anderson et al (1940) found that a 1% sodium chloride drink restores the electrolyte changes of adrenalectomised rats to normal. Francois Morel (1951) found that sodium excretion of adrenalectomised rats kept on 0.9% sodium chloride drink was not significantly different from normal controls. The usual procedure in most methods of assay discussed before was to withdraw the saline drink several hours before the beginning of the assay. This procedure would tend to cause a certain degree of sodium depletion which would allow the sodium retaining effect of the mineralo-corticoids to be more marked. There seems to be a critical degree of sodium depletion beyond which the animal is adversely affected and severe symptoms of cortical insufficiency ensues; Loeb (1932), Thorn et al (1936, 1937, 1938).

In the method reported here five hours withdrawal of the saline drink was thought to be sufficient.

5. The sodium load.

Sodium depletion, if severe,

may/

may lead to convulsions and death (Hays et al, 1945).

On the other hand, if the plasma osmotic pressure of adrenalectomised rats is raised by injecting hypertonic sodium chloride solutions, mineralocorticoids hardly inhibit sodium excretion by the kidneys (Morel, 1951).

Dorfman et al (1947) made the same observation. When a sodium load of 1.75 per cent sodium chloride containing a total of 17.5 mg. of the salt was administered a small degree of sodium retention followed the injection of 1.0 mg. DOC to adrenalectomised rats.

The ratio $\frac{\text{control sodium excretion}}{\text{DOC treated sodium excretion}}$ was 1.4.

When 2.0 mg. DOC was injected under the same conditions, the ratio was 1.43.

The same authors used an isotonic load with a total content of 3.25 mg. sodium chloride and found that the rate of sodium retention was much higher. The ratio was for doses of 1.0 mg. and 2.0 mg. DOC, 3.63 and 8.12 respectively.

Hays (1952) found that adrenalectomised rats sixteen hours after the operation have their maximum rate/

rate of diuresis when given a load of 0.2 per cent sodium chloride. On the 7th day after adrenalectomy, the same author found maximum diuresis to occur with sodium chloride loads of 0.5 per cent to 1.25 per cent.

Nagareda et al(1951) reported that a water load produced a significant adrenal ascorbic acid depletion. Huge loads of saline did not produce any depletion.

The sodium load given to adrenalectomised animals, if near isotonicity, would thus cause increase sensitisation of the animal to sodium retaining effect of DOCA, increase diuresis and cause little or no stress.

The ideal conditions for assays thus seems to be a mild degree of cortical insufficiency accompanied by a moderate degree of sodium depletion and the supply of an isotonic or slightly hypotonic load. This load should contain enough sodium to replenish any marked degree of sodium depletion and not too much to tax the renal tubules and upset the critical equilibrium established.

Dividing the load into portions was therefore thought to be a method of achieving some of these aims/

aims. By giving the first load as a solution of 0.397 per cent sodium chloride and 5 per cent glucose the tonicity of the load was presumed to be in the optimal range. 4.0 ml. of this solution injected subcutaneously to every animal thus supplying each animal with 6.25 mg. sodium (or 15.897 mg. sodium chloride). This animal was expected just to replenish the degree of sodium depletion established by withdrawing the food 11 hours and the sodium drink 5 hours, beforehand. The glucose was acting as a source of energy over the prolonged period of fasting. After the injection of the first load the animals were left for a period of 2 hours. It was observed that they secreted an appreciable volume of urine in this period and, what is more important, that their general condition improved considerably. Thus the assay was begun with animals in a fit condition and it was rarely necessary to discard any of them.

At the end of the 2 hour period, the bladder was emptied and the second portion of the load solution injected with or without, the mineralo-corticoid to be tested. The second portion of the load was 1.0ml. of a solution of 0.7948 per cent sodium chloride and

.0356 per cent potassium chloride, that is 3.125 mg. sodium and 0.2 mg. potassium.

6. Control values.

Deming and Luetscher (1950), Dorfman et al (1947), Spencer (1950), Simpson and Tait (1950) and Singer and Venning (1953), reported on the wide range of individual as well as group variation in sodium excretion in adrenalectomised animals. Attempts at standardising procedure and conditions did not diminish the variations between different groups studied on different days (Singer and Venning, 1953). Spencer attempted to overcome this difficulty by expressing the test and standard values as a ratio of the control value obtained from the same animal. As reported before, in my experiments I was not able to use the same animal repeatedly. The method of increasing the number of animals in each group and including a group of controls in each assay followed by most of the other workers was found more suitable and less laborious.

7. Vehicle for introducing steroids.

Deming and Luetscher (1950) made a study of the possible vehicles appropriate for administering cortical steroids in such assays. Using 150 g./

150 g. male rats, they preferred ethanol solutions. Simpson and Tait (1950) found that in experiments which last 2 hours only, ethanol was the ideal vehicle as it allowed for rapid absorption. Ethanol solutions of 10 per cent - 20 per cent are not very good solvents for some steroids, and with larger quantities of the steroid a larger volume had to be used. Propylene glycol is a more powerful solvent and does not affect the animal in the doses given. 0.1 ml. of propylene glycol was chosen as the solvent of choice, larger doses of 0.2 and 0.25 ml. have also been used occasionally without untoward effect on the assays.

8. Urine collection.

An incomplete urine collection is one of the most important variables in this type of assay which measures the excretion of one electrolyte only. It is important to evacuate the bladder completely at the beginning and at the end of the collection. Kagawa et al (1952) proposed a complicated method to achieve this end. The animals were given whiffs of ether and suprapublic massage to empty the bladder. The urethra was then ligated, the bladder dissected out, the urine collected and the/

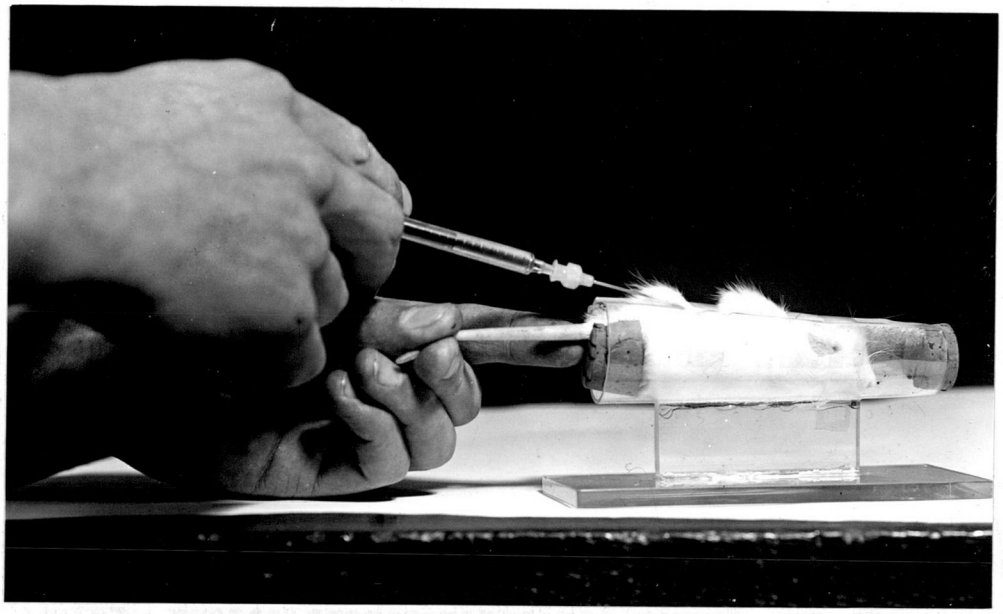
the bladder washed. Beginning the urine collection by ether anaesthesia as suggested by Kagawa did not prove to be an advisable procedure in young animals. An easier method was found which consisted in applying moderate traction on the tail. This can be repeated several times and in the majority of animals is quite sufficient to evacuate the bladder completely. To ensure complete evacuation of the bladder in all animals gentle suprapubic massage was used. The very few animals which still refused to urinate were put aside for one minute, or two; usually they urinated then, otherwise the whole procedure was repeated. At the end of the assay, a polythene wash bottle was used to sprinkle into each metabolic beaker about ten drops of ether. Just as the animal was getting drowsy, it was picked up and the supra--pubic region massaged firmly against the edge of the beaker, the urine being washed down into the beaker with a fine jet of distilled water.

In a number of experiments, the rats were killed and the bladder dissected to test the efficiency of these methods of evacuating the bladder. It was found that the bladder had always been emptied following either of the two methods.

A proposed method of assay/

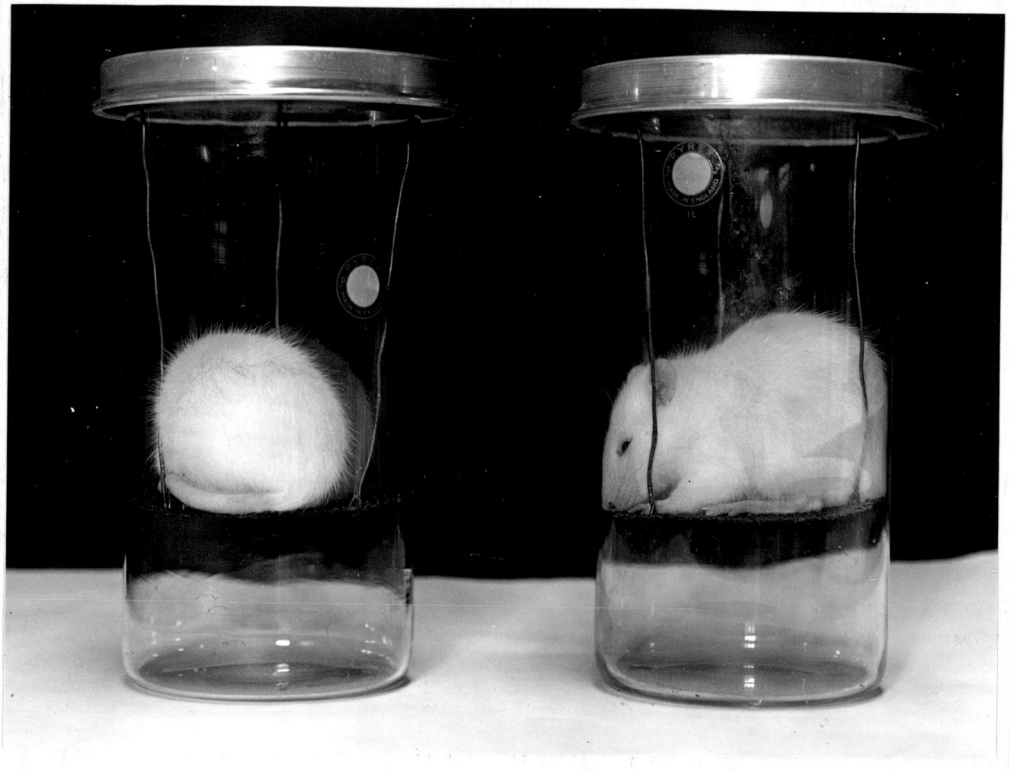
Figure 4

"A"



Apparatus for giving injections.

"B"



Apparatus for collecting urine.

A proposed method of assay.

Method:

Young female white rats of 50-100 g. were used. The animals were adrenalectomised under ether anaesthesia. After adrenalectomy they were kept on a drinking solution containing 1 per cent sodium chloride and 5 per cent glucose and fed on rat cubes. The temperature of the room was kept constant throughout the whole experiment at 22-24°C.

On the fourth day after adrenalectomy, feeding was stopped at 11 p.m.; the drinking solution continued till 5 a.m. next morning, when it was replaced by 5 per cent glucose only. At 10 a.m. the animals were injected with solution L. (6.25 mg. sodium as chloride in 4 ml. 5 per cent glucose). 4 ml. of this solution were injected subcutaneously to each animal. (Fig.4).

The amount of handling was cut down to the minimum possible; the animals picked by the tail and brought near to a polythene container of a cone shape with a slit on the upper surface. Once they have run in, the container was closed with a rubber stopper with a corresponding slit which allowed the tail/

tail to stay out and the needle (3 inches long) was introduced near the tail and pushed subcutaneously towards the shoulder region; then the injection was given slowly.

One animal is injected from each group in turn. The groups, each containing about eight animals, were arranged so that animals with corresponding weights were evenly distributed in the different groups.

At 12 noon, the second load and the corticoids were administered:-

A rat from each group successively, in the same order as they were given the first injection, was used.

Each rat was put in the container and the tail pulled to evacuate the bladder in the manner described before.

One ml. of the second load was then injected subcutaneously in the same way as the first load. The second load contained 3.125 mg. sodium and 0.2mg. potassium per ml. At the same time as the load was injected, a tuberculin syringe with a fine needle was used to administer the corticoid in 0.1 ml. propylene glycol subcutaneously. The control group received the propylene glycol alone.

Once/

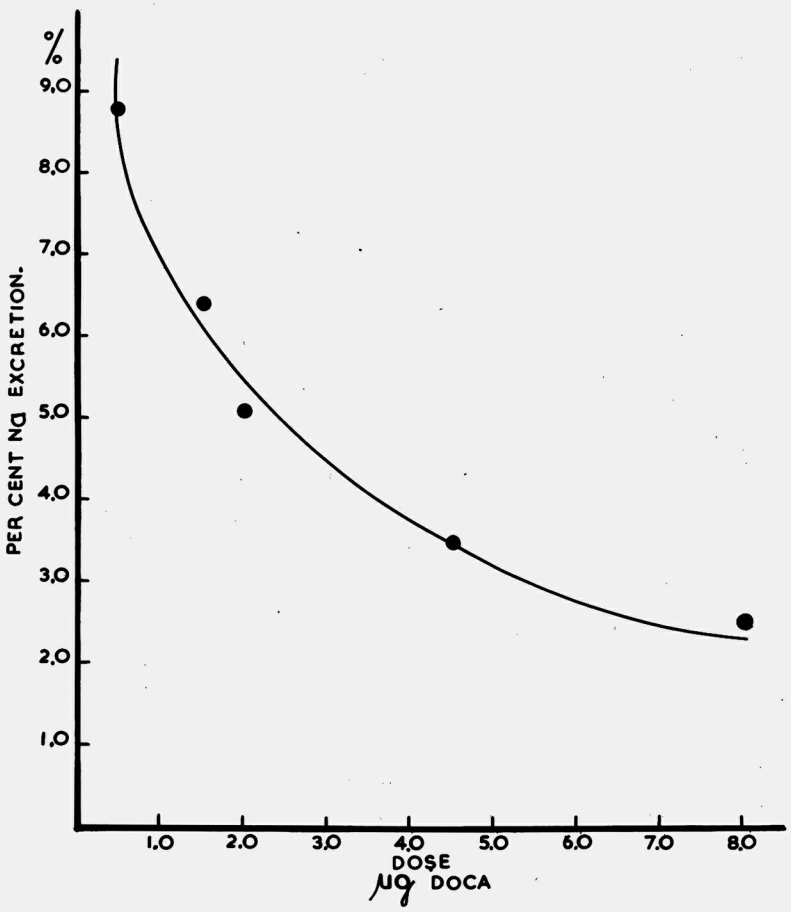
Once a rat was injected it was carried directly to its metabolism beaker in the same order as they were injected.

These metabolism beakers were of one litre size and made of pyrex glass. A tightly fitting wire mesh platform was suspended from the rim of each beaker by wire hangers. The hangers allow the platform to hang two-thirds of the way down the beaker. Before each platform was used it was waxed in pure paraffin wax and washed with cold distilled water. All the apparatus was kept thoroughly salt free. Each metabolic beaker had an aluminium lid with holes for ventilation.

Six hours after the injections, the urine collection was ended in the same order as the animals were handled all through the experiment.

The bladder of each rat was emptied, using ether drops and massage as previously described. Any faeces which may be lying on the wire mesh platform were removed by forceps and the beaker and platform washed thoroughly with 20 ml. of distilled water four times, and the washings made up to 100 ml. This was filtered,using ashless acid-washed filter paper, and the amount of sodium estimated by the flame/

Figure 5.



Dose response curve for DOCA (µg)

(TABLE VII)

flame photometer.

The rats were kept on cubes and tap water and almost invariably died all in eight days after the withdrawal of the saline drink.

TABLE VII.

Effect of DOCA on Sodium Excretion in the Urine of Adrenalectomised Rats.

Dose	0.5µg.	1.5µg.	2.0µg.	4.5µg.	8.0µg.
No. of rats.	15	15	16	15	16
Mean Na excretion (% of controls) ± S.D. of differences[1].	88.1±23	64.1±20.7	51.3±16.9	35.2±17.4	25.0±15

[1] Standard deviations of differences between test and control groups.

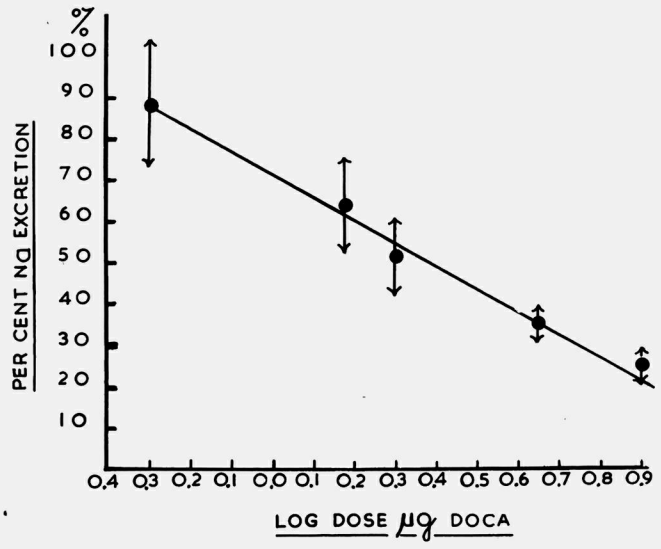
Test of significance: Between control group and 2.0µg. $P < 0.01$
Between 1.5µg. and 4.5µg. $P < 0.05$.
Between 2.0µg. and 8.0µg. $P < 0.02$.

TABLE VIII.

Analysis of Variance.

Adjustment for mean - 209,852.4			
Nature of variance	D.F.	Sum of squares	Mean square
Regression	1	37268.3	
Deviations from regression.	3	418.7	139.57
Between doses	4	37687.0	
Within doses	72	104468.46	1450.95
Total	76	142155.49	

Figure 6.



Dose response curve for DOCA (log dose).

(TABLE VII)

Results

Tables VII and VIII give the figures for a dose response curve and their statistical analysis.

The effects on sodium excretion are expressed as per cent of the mean control values. The deviations from linearity are very small. The slope $b = 53.3 \pm 10.52$ giving a t value of 5.07 indicating that the response is dependent on the dose at a probability level of < 0.001. The pricision index however, is high:-

$$\lambda = 0.714 \quad \left(\lambda = \frac{\text{standard deviation}}{\text{slope}} \right)$$

A dose of 2.0 µg. DOCA gave a response significantly different from the control value ($P < 0.01$).

Because of the flatness of the curve, the dose had to be increased by a relatively high factor to achieve mean responses differing significantly. The mean response for a dose of 1.5 µg. DOCA differs from that for a dose of 4.5 µg. at a probability of $P < 0.05$. (Figs.5 and 6).

The variations between different blocks of rats inspite of attempts at standardising the conditions of the experiments necessitates the inclusion of a group/

group of control animals in each assay and the expression of the responses of the test and standard animals as a percentage of the mean response of the control group.

Because of variations in slope it is necessary to have two groups injected with known doses of the standard included in every assay. Each group should include 10-15 rats.

Applications.

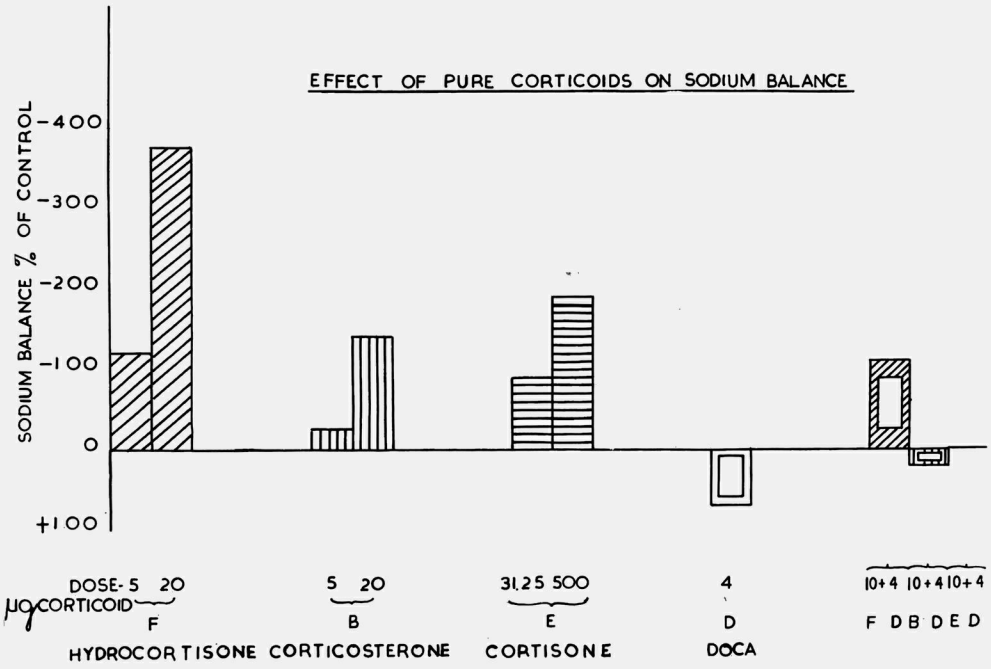
A. The effect of pure cortical steroids on sodium excretion.

Method. The same method of assay was used to investigate the effect of different cortical steroids on sodium excretion.

Corticosterone (compound B), 17-hydroxy-11-dehydro corticosterone (compound E) and 17-hydroxy-corticosterone (compound F) were tested. The different steroids were dissolved in propylene glycol and given in 0.1 ml. of solution per each rat. Urine collection was over 6 hours.

Results. As is shown in Table IX, all the cortical steroids tested with an oxygen at C^{11} have a tendency to increase sodium urinary excretion under the/

Figure 7.



Effect of different doses of the corticoids and 10 μg of each in combination with 4 μg DOCA.

(TABLE IX)

the conditions of the test. There was a tendency with all the steroids for sodium excretion to increase with increasing the dose. Small doses of DOCA (e.g. 2.0 and 4.0 µg. per rat) given to a group of animals run concurrently with some of the experiments, showed a marked sodium retention as usual.

When DOCA was administered in conjunction with steroids causing sodium diuresis antagonism between the two steroids was observed in every case. It may be of interest, that a steroid like compound F which showed the greatest potency as a sodium diuretic when given alone, also showed the greatest degree of antagonism when administered with DOCA.

It was thought possible that the potency of the sodium diuretic steroids can be assessed roughly by their power to antagonise the sodium retaining effect of DOCA. 10.0 µg. of each of the sodium diuretic steroids was administered in conjunction with 4.0 µg. of DOCA. The results obtained seem to indicate that the potency of these compounds is in this descending order: Compound F
Compound E
Compound B

To/

To investigate whether such an antagonism does in fact have any quantitative nature several doses of DOCA were administered in conjunction with two doses of compound E. From Table IX it is seen that, while 4.0 µg. DOCA was about enough to neutralise the sodium diuretic effect of 10.0 µg. compound E and 8.0 µg. DOCA did the same to 31.25 µg. compound E, 2.0 µg. DOCA failed to neutralise 31.25 µg. compound E. (Fig.7).

Compound B which has the weakest potency as a sodium diuretic did in fact, cause a non-significent degree of sodium retention in one experiment when administered in a dose of 20.0 µg. per animal. In the other eight experiments carried out on this compound there was an increase in sodium excretion or an antagonism to the sodium retaining power of DOCA.

TABLE IX/

TABLE IX.

Pure Corticosteroids and Sodium Urinary Excretion.

Steroid	Dose in µg.	DOCA µg.	No. of animals	Sodium excretion[1] per cent ± S.E.	DOCA run concurrently. Dose in µg.	No. of animals	Sodium excretion[1] per cent ± S.E.
Comp.F.	5.0		15	219.1 ± 25.01			
	20.0		14	474.3 ± 12.51			
	10.0	+4.0	8	210.3 ± 31.17			
Comp.B.	5.0		14	128.1 ± 24.58			
	10.0		7	135.0 ± 17.0	4.0	7	30 ± 9.7
	20.0		13	240.8 ± 41.97	4.0	8	34 ± 10.1
	40.0		7	189.8 ± 29.49			
	10.0	+4.0	19	80.5 ± 13.01			
Comp.E.	31.25		8	187.0 ± 30.06			
	125.0		7	203.4 ± 24.42			
	500.0		8	290.55 ± 44.84			
	10.0	+4.0	11	112.15 ± 14.96			
	31.25	+2.0	7	163.14 ± 21.36	2.0	6	25.8 ± 8.39
	31.25	+8.0	6	103.14 ± 10.57			

[1] Sodium excretion expressed as per cent of mean control.

B. The effect of extracts of corticoids from adrenal venous blood on sodium excretion.

1. Dog

Methods:

a. Collecting adrenal venous blood. The operative technique described by Vogt (1943) was used. Adult male dogs of about 14 kg. body weight were anaesthetised with ether until a chloralose solution (70-80 mg. per kg. body weight) was infused into the femoral vein.

A tracheal cannula was introduced and an artificial respiration pump kept nearby in case of respiratory failure.

The adrenal vein on the left side was located next. All tributaries from surrounding tissues, apart from the adrenal gland itself, were ligated. All bleeding points were firmly secured and a heparin solution infused into the femoral vein (5000 units in saline). The carotid artery was then cannulated and connected to a manometer to record changes in blood pressure on a kymograph.

An L shaped cannula was then connected to a polythene tube and the cannula introduced into the adrenal/

adrenal vein. The vena caval end of this vein was then ligated. Blood flowing exclusively from the left adrenal gland was thus collected into 50 ml. centrifuge tubes kept in an ice bath. The rate of blood flow was recorded. The blood was centrifuged and the plasma pipetted as soon as collection of each tube ended. Arterial blood from the carotid artery was collected before killing the dog and the weight of both adrenal glands obtained. In dog C the splanchnic nerves were severed before blood collections started.

b. Chemical extraction. The method devised by Bush (1951) as reported by Bibile (1953) was used. A mixture of ether and ethyl acetate 1:3 is prepared from freshly distilled stocks. Each volume of plasma was extracted by shaking it with its own volume of the mixture. After centrifuging, the non-watery phase is pipetted into a round-bottomed flask. The extraction process is repeated four times. The collected supernatants are evaporated in vacuo, the temperature of the bath being around 40°C. until a thin film of fluid is left in the flask.

A volume of petroleum ether corresponding to the initial volume of the plasma is added to the flask and the sides of the flask are well scraped

by/

by a glass rod. A separating funnel is prepared containing $\frac{5}{3}$ of the initial plasma volume of petroleum ether and the contents of the flask is added to the funnel. The evaporating flask is further washed and thoroughly scraped with 70 per cent redistilled ethyl alcohol in water, $\frac{1}{7}$ of the initial plasma volume being used. The alcoholic washing is repeated and the alcohol added to the funnel. The contents of the funnel are shaken thoroughly and allowed to separate and the alcoholic phase is then collected in a flask. The whole process of alcoholic extraction is repeated twice. The collected alcoholic extract is then evaporated in vacuo, and the residue dissolved in propylene glycol.

When DOCA was to be added to the plasma before extraction this was carried out in the following way. A solution of DOCA in 40 per cent alcohol was prepared as concentrated as possible and the solution was added to the plasma before extraction started. The same volume of a 40 per cent alcohol was added to the control plasma.

The concentration of the final extract in propylene glycol was the same as that used for the adrenal/

adrenal sample. The arterial extract was used as a vehicle for administering the standard doses of DOCA in the assay. In this way, the presence of active materials in the plasma not produced by the left adrenal and which might affect the assay in an unpredictable manner, was compensated for.

Arterial plasma from control dogs has been obtained and extracted in the same way. These extracts have been assayed without the addition of any cortical steroids.

c. Method of assay. The previously described method of assay was used to test the mineralo-cortical activity of arterial plasma and adrenal venous plasma of dogs. 0.1 ml. of propylene glycol extract corresponded to between 2.0 and 2.25 ml. adrenal plasma. The standard doses of DOCA were given in the final extract of the same volume of arterial plasma.

TABLE X

TABLE X

Mineralo-cortical activity of Dog Arterial Plasma.

Expt. No.	No. of rats in each group.	Mean sodium excretion of test group as % of mean of control group ± S.D. of differences[1]		
		Extract of 0.75 ml. arterial plasma.	4.0 µg. DOCA	Extract of 0.75 ml. arterial plasma containing 4.0 µg. DOCA[2]
1	7	103.0 ± 58	61.02 ± 16	66.9 ± 16
2	7	115.0 ± 29.6	43.9 ± 20.9	64.0 ± 20.8

[1] Standard deviations of differences between test and control groups.

[2] The DOCA was added to the arterial plasma before extraction.

TABLE XI.

Mineralo-cortical activity of Dog Adrenal Venous Plasma.

Dog	Assay	Vol. of plasma per rat ml.	No. of animals/ group.	Mean Na excretion of test group as % of mean control group ± S.D. of differences.	DOC-equivalent µg./ ml. plasma.	Significance of difference from control (P)
A	I	2.0[1]	8	78.98 ± 20.14	0.675	0.3 -0.4
	II	2.0[1]	7	64.2 ± 21.93	0.659	0.1-0.2
B	I	2.0[2]	7	102.4	0.0	
	II	2.0[2]	7	96.3	0.0	
C[4]	I	2.25[3]	9	75.06 ± 22.32	0.33	

[1] 2.0 ml. of the adrenal plasma collected over 1.98min.

[2] 2.0 ml. " " " " " " 1.32min.

[3] 2.25ml. " " " " " " 3.06min.

[4] The splanchnic nerves were severed before the collection.

Results. Extracts of arterial plasma did not cause any sodium retention (Table X). If anything, a tendency to enhance sodium excretion was observed, but this was not significant. When arterial plasma was extracted after the addition to the plasma of 4.0 µg. DOCA per 0.1 ml. of the final extract, this extract caused sodium retention to an extent slightly less (differences non-significant) than that produced by 4.0 µg. DOCA alone. Because of the reported sodium diuretic effect of pure arterial plasma extract these differences are more likely to be due to sodium diuretic substances in the plasma than to be due to incomplete recovery of the DOCA added after extraction.

Extracts of adrenal venous plasma. The sodium retaining activity of the samples examined could not be established (Table XI). When extracts of 2.0 ml. plasma were injected per rat the sodium retaining activity was very near the threshold of the method of assay employed. The sodium retention caused by this extract was not significant. The calculated activity therefore, is only a rough estimation.

A comforting feature, however, was that when the same sample was assayed twice, the results agreed very/

very well with each other. When the splanchnic nerves had been severed (dog C) and the rate of venous flow from the adrenal vein was slowed down, no increase of mineralo-cortical activity was observed.

In order to obtain as high a concentration of corticoids in the adrenal effluent as possible, the blood obtained at higher blood pressures, and consequently a rapid rate of flow, was discarded. Only the latter samples, when blood loss diminished the pressure and flow, were used.

When mineralo-cortical activity was calculated as µg. DOCA per gram of adrenal gland per minute (Table XII) one feature, whose significance it is impossible to assess with the available data, was observed. Dog A, in which the adrenal sample was collected while the systolic pressure was almost double that in the other dogs, gave the highest sodium retaining activity.

If this observation can be sustained by more significant results, it may be due either to greater liberation of sodium retaining hormones at higher blood pressure, or smaller liberation of sodium diuretic hormones.

TABLE XII/

TABLE X11.

Sodium retaining activity of dog adrenal venous plasma.

Dog	Sodium retaining activity µg. DOCA per g. per min.	Mean systolic pressure during sample taking
A	0.95	64.0
B	0.0	35.0
C	0.44	32.0

2. Rabbit

Methods:

a. Collection of adrenal plasma was carried out according to the method described by Vogt (1943). Rabbits of 2.5-3.0 kg. weight were anaesthetised with ether followed by chloralose infused into the jugular vein. Through a median abdominal incision the animal was eviscerated, care being taken to empty the blood contents of the viscera into the animal's circulation before ligating the portal vessels. An artificial respiration pump was used whenever required. Ligatures were then tied around all vessels draining into the/

the inferior vena cava except the adrenal veins. Venules draining the fatty tissue surrounding the kidneys and those draining the vertebral column were included.

A loose ligature was placed around the inferior vena cava above the entrance of the right adrenal veins. The aorta was ligated below the origin of the renal arteries, and a cannula introduced into the vena cava below the level of the kidneys. Heparin was then administered through the jugular vein and a carotid cannula for blood pressure recording introduced. The ligature around the upper part of the vena cava was tied and the blood allowed to run into the cannula for the collection of samples. The blood pressure was maintained as constant as possible by infusing heparinised blood collected from another rabbit.

The volume and time of each collection was recorded.

At the end of the experiment the two adrenal glands were dissected out and weighed.

In the second experiment 10.0 units of ACTH dissolved in 40 ml. saline were warmed to body temperature, and infused through the jugular cannula at the rate of 2.0 ml. per minute. A blood collection was carried/

carried out during the last phase of the infusion and after.

b. Extracting the rabbit plasma.

Method: The same method as that described for the dog was used also for the rabbit.

Because of the impossibility of collecting enough arterial plasma from the same animal to use its extract as a vehicle for introducing the standard doses of DOCA, these standard doses were given in 0.1 propylene glycol alone.

A sample of arterial plasma collected from two rabbits was assayed after extraction, (Table XIII).

c. The method of assay previously described was used, the extract of plasma given to the test group in 0.1 ml. propylene glycol per rat containing the extract of 1.7-3.5 ml. of original adrenal plasma.

Results. In Experiment 1, plasma extract of 3 ml. collected over a period of 1.6 minutes were administered per rat in the assay; significant sodium retention was observed (Table XIII). On the other hand, 1.7 ml. plasma collected over a period of 2.8 minutes had no significant effect (Experiment 2). Another sample of plasma collected from this same second rabbit after infusion of 10 units of ACTH did/

TABLE XIII

Mineralo-cortical activity of rabbit plasma.

Experiment.	No. of animals per group	Extracted plasma administered per rat		Mean sodium excretion of test and standard groups as per cent of mean control.				Estimated mineralo-cortical activity of plasma as µg DOCA per ml plasma.
		Vol. of plasma ml	Collection time in mins.	Group given plasma	Significance from control	Group given 2.0µg DOCA	Significance from control P	
I Adrenal venous plasma	11	3.0	1.62	41.6	$P < 0.001$	47.0	$P < 0.001$	1.2
II Adrenal venous plasma	16	1.7	2.85	89.7	$P > 0.4$	50.2	$P < 0.001$	0.0
Arterial plasma after ACTH	16	3.5	3.05	79.8	$P < 0.2$	50.2	$P < 0.001$	0.0
Arterial plasma	8	3.0		129.9	$P = 0.5$			0.0

did not cause any significant change in the sodium retaining effect of the plasma. This sample has been assayed in quantities corresponding to double the collection time used in the samples before ACTH administration (3.5 ml. plasma collected over 3.05 minutes). Inspite of that, no significant sodium retention was observed.

Arterial plasma from the rabbit showed the same tendency, like that from the dog, to cause slight sodium diuresis.

For the first rabbit mineralo-cortical activity output was calculated to be 2.2 µg. DOCA-equivalent per gram of adrenal gland per min.

DISCUSSION/

DISCUSSION

Method of Assay.

The method reported here for assaying mineralocortical activity offers certain advantages.

a. Simplicity of procedure. It has been the aim throughout this work to develop this method along lines which permit its adoption as a routine method of investigation not requiring elaborate technical facilities or excessive labour-hours.

b. Reasonable range of sensitivity. Significant sodium retention was achieved by 2.0 µg. DOCA doses in experiments where 10-15 rats per group were employed. 1.0 µg. DOCA gave significant sodium retention in one experiment (P = .03) but the results were not reproducible in other experiments. The highest limit used in the experiments reported here was 8.0 µg. DOCA. In other experiments, the effect of 32 µg. DOCA was greater than that of 8 µg. though the difference was not significant.

c. The ability to differentiate between mineralo-corticoids and corticoids with an oxygen atom at C_{11} (11-oxycorticoids).

The reported method, on the other hand, suffers from/

from some serious handicaps.

The index of precision is very high = 0.714. This represents a low degree of accuracy which seems to be the unavoidable price of simplicity of procedure. As a result of this inaccuracy it is necessary to increase doses by a high factor (3-4) to achieve significant differences in response.

Assay of pure cortical steroids.

When this method was used in a comparative study of the action of some of the known corticosteroids on electrolyte excretion, the results like those of Marcus et al (1952) and Singer et al (1953) were obtained. With the present method as well as with these methods there is a demonstrable qualitative difference between DOCA and the 11-oxycorticoids on the excretion of sodium by adrenalectomised animals. The 11-oxycorticoids cause an increase in sodium excretion instead of a decrease.

Simpson and Tait's (1952) method on the other hand, does not reveal such qualitative differences. All the active corticoids tested caused sodium retention the only difference being a quantitative one. The $\frac{Na}{K}$ ratio dropped in all cases.

Singer et al explained this difference between their/

their results and that of Simpson and Tait by three possible causes.

a. Simpson and Tait were measuring the variations in sodium potassium ratio. Thus, even if sodium excretion is enhanced by the glyco-corticoids, the potassium excretion may be enhanced to a greater extent and thus a diminished ratio is still observed.

b. The urine collection was carried out over a period of two hours only in Simpson and Tait's method instead of 5-6 hours as in the other methods.

c. Simpson and Tait used a much smaller sodium load than that used by the other methods. Simpson and Tait considered the two hours' collection a necessity to include the steroids which have a short-lived activity on mineral metabolism as is the case with cortisone. The minute sodium load administered in their method served to approach a true balance experiment in which the electrolyte composition of the tissues was not changed by the load.

Which ever of the suggested causes is the explanation of this discrepancy, it is obvious that the responses measured in these two sets of tests are not identical. It is of importance to assess the significance of this difference in response and relate/

relate it to the complex changes initiated by cortical hormones in the electrolyte metabolism of adrenal deficient animals. The three possible causes of this discrepancy are discussed below.

a. The effect of measuring the $\frac{Na}{K}$ ratio in Simpson and Tait's method instead of the sodium excretion by itself is difficult to assess.

Dorfman (1949) found potassium excretion in the urine of adrenalectomised rats to increase with the increase of the dose of DOC injected. In the experiments reported here, potassium excretion reached a maximum with 2.5 µg. DOCA and then started to drop. The $\frac{Na}{K}$ ratio was calculated for the cortisone experiments reported previously (page 72). It was found that, under the conditions of this method of assay, the ratio for the cortisone-treated group was always higher than that for the control group. The highest value was that obtained with doses of 31.25 µg. cortisone and the ratio then dropped again with higher doses but remained above the control level even with doses of 500 µg. of cortisone. The potassium excretion, however, was found to increase with the increase of the dose except with the smaller doses (31.25 µg.) when no increase was demonstrable. As the conditions of the reported experiments are

different/

different from those of Simpson and Tait's it is difficult to draw conclusions.

b. The difference in the sodium loads does not seem to be a likely cause of the inversion of the response so long as the load is within physiological boundaries.

An adrenalectomised animal requires a large amount of sodium to keep it in a state of balance approaching physiological conditions. Richter, (1938-1941) found that the adrenalectomised rats select for drink exclusively fluids containing sodium salts.

Anderson et al (1940) found that the optimal daily intake of sodium chloride for an adult adrenalectomised rat was from 650 to 940 mg. A level of 339 mg. was not sufficient for growth or survival.

It is obvious that in tests carried out on adrenalectomised rats deprived of sodium intake for several hours the sodium loads employed by all the discussed methods fall well within physiological limits.

c. The period of urinary collection may however, play a more dominant role.

Davis/

Davis and Howell (1953) pointed out that an important consideration which has often been neglected in studying water and electrolyte balances in response to cortisone is the role of glomerular filtration. This applied to dogs and rats. In normal dogs observed over two days, the authors found that cortisone and ACTH caused increased salt loss only in those dogs in which the glomerular filtration rate was raised by the hormone.

The rise in glomerular filtration and renal plasma flow was most profound with cortisone treatment and least with DOCA treatment.

Roberts and Pitts (1952), in their acute experiments on dogs demonstrating the sodium retaining power of cortisone, collected urine over a period of 2-3 hours only, after the administration of cortisone. The glomerular filtration rate and plasma flow were not significantly altered during this period. The changes in the subsequent period were not investigated.

Another factor which may be involved in a six hours urinary collection and not in a shorter period has been indicated by Bloodworth (1952). In normal dogs, he found that administration of cortical extract/

extract for three to six hours caused a transfer of fluids from the intracellular to the extracellular compartments. When DOCA was used over the same period no such transfer was observed.

The possibility that the mineralo-cortical activity of cortisone is a more complex phenomenon than can be estimated in a brief period of observation of a single variable need further investigation.

The different factors involved in the mineralocortical activity of the different corticoids need not all be affected simultaneously or act all in the same direction.

Simpson and Tait found a decrease in the urinary $\frac{Na}{K}$ ratio after giving cortisone only when they used a two hours collection period. Such a drop in the ratio was not obtainable with a six hours collection.

DOCA too, seems to have a variety of actions when its effects are followed over a prolonged period. While its sodium retention effect is easily demonstrable a few hours after administration, its cold protection effect is demonstrable only twenty-four hours after administration (Vogt, 1943). The same/

same holds for many of its effects on carbohydrate metabolism. Verzar and Wang (1949, 1950).

The so-called short-lived activity of cortisone on mineralo metabolism may be thus an inaccurate description of an activity which is short-lived only in the sense that it is soon to be supplanted by other effects which, while reversing its direction, may still be due to the same fundamental action on cellular metabolism.

It seems, however, premature to try to arrive at any convincing conclusion whether the parallelism demonstrated by Simpson and Tait does represent an actual parallelism of the fundamental mineral properties of the cortico-steroids or a mere parallelism of a passing phase in the complex actions of two different steroids.

The qualitative differences between 11-oxy-corticoids and DOCA-like corticoids have been confirmed by several workers.

Ingle (1940) demonstrated such a difference in the action of cortisone and DOCA on the muscle performance and the rate of growth of adrenalectomised rats./

rats. In the case of the rate of growth Ingle found a definite antagonism between the actions of the two corticoids.

Thorn et al (1941) suggested that a qualitative difference between the action of these two groups of corticoids might be the reason why marked sodium retention, increased plasma volume and hypertension do not follow prolonged administration of cortical extracts as they do follow the administration of DOCA.

Wells and Kendall (1940a,b,c) found that DOCA maintained the growth of normal rats at the normal level or above it, while corticosterone retarded growth.

DOCA was the only steroid to cause a reduction of the serum potassium level of normal rats treated with cortical hormones.

In adrenalectomised phlorhizinized rats, cortisone and corticosterone successfully maintained the animals in excellent condition and thus prolonged the survival time. DOCA had no such effect.

The authors conclude that their observations indicate differences in the effects of these compounds which/

which cannot be explained by variations in the rate of absorption, utilisation, destruction or excretion. The differences appeared to be due to differences in structure.

The authors observe that the retarding influence of some compounds on growth could conceal a beneficial effect of others. This was confirmed by Ingle (1940.

Verzar (1950) on the other hand, conceived the functions of the corticoids to be basically a unitary function common to all. Wirz (1951) supported this view by his observations. He treated cortical deficiency in rats with DOC and cortisone. If given once a day, the power of cortisone to restore the depressed plasma sodium was weaker than that of DOC. When the same dose was divided into eight hourly doses the potency of the two corticoids became equal.

While these observations indicate the almost complete parallelism that can be achieved between the actions of different corticoids under given conditions, they do not abolish the established differences observed under other conditions.

The/

The conception of Verzar seems to be open to criticism just as much as the opposite conception of a definite separation of the corticoids into glyco-corticoids and mineralo-corticoids. Both conceptions are too rigid to accommodate the known facts about the behaviour of the different corticoids and their combinations.

These observations seem to indicate that the functions of the different corticoids are of a complementary nature. The parallelism or the antagonism is incidental and depends on several factors. The state of balance of the organism, and the period of observation seem to be only two of these factors. The response chosen for observation and the speed of action of the corticoid obviously play their roles too.

The complementary nature of the actions of different corticoids is illustrated by two further observations.

a. The functions of the pure corticoids are not only complementary among themselves but seem to maintain the same relationship with the functions of other steroids excreted by the gonads. Many of the/

the effects produced by progesterone are known to be produced also by desoxycorticosterone. Adrenalectomy has a suppressing effect on gonadal activity which can be corrected by injecting cortical extracts. The gonads also are thought to possess cortin-like activity, progesterone being a notable example in this connection, (Parkes, 1945). This relationship does not seem to be a simple one-direction mechanism. Selye (1944) gives a list of adrenal steroids which possess either androgenic, oestrogenic or progestational properties.

b. During metabolic processes physiologically active steroids may give rise to byproducts which are physiologically active in the reverse direction.

Emmens (1941) found ethisterone to have a progesterone-like action when applied directly to the uterine endometrium. Its activity is almost as great as that of progesterone. When administered to the whole animal ethisterone undergoes metabolic changes and its function is reversed and the new product has an oestrogenic effect.

Pincus and Thiemann (1949) and Jacobsen and Pincus (1951) reported on the ability of the adrenal to attach an/

an oxygen atom to C_{11} in 11-desoxycorticoids. Pfeffer et al (1952) confirmed this observation in man.

A new corticoid with a very high mineralo-activity has been isolated by Simpson and Tait (1953). Luetscher and Johnson (1953), Knauff et al (1953) and Mattox et al (1953).

Simpson et al (1953) found the new corticoid to be 30-100 times as active as DOCA. Mattox hydrolised its acetate with citrus acetylase and obtained a product 85 times as active as DOCA.

A new field of investigations will be open when this compound is available.

Comparative studies which lead to fuller understanding of the nature of the function of the different corticoids can be expected when the natural mineralo-corticoids take the place of the synthetic compound DOCA. The latter has hitherto been presumed to act precisely like the natural hormone without any concrete evidence that it does in fact, have exactly the same properties.

Plasma mineralo-cortical activity.

No sodium retaining activity was demonstrable in arterial plasma of the dog or the rabbit. If anything, extracts of arterial plasma had a mild sodium diuretic effect.

Adrenal venous plasma, in the dog, had no significant sodium retaining activity. The highest equivalent obtained was of the order of 0.9 µg. DOCA per gram adrenal gland per minute.

Spencer (1950), in one experiment estimated the sodium retaining activity of dog adrenal venous serum to be 4.0 µg. DOCA equivalent per ml. Arterial blood from the dog had no activity and extracts of a large volume of this blood had very little activity.

Simpson, Tait and Bush (1952) demonstrated the presence of a highly active mineralo-corticoid in the secretion of the adrenal gland of both dog and monkey. Using chemical extraction and paper chromatography they could separate this active substance in the section of the paper known to contain cortisone. When the mineralo-cortical activities of all the fractions are added together the dog adrenal secretion is estimated to have 20·22 µg. desoxycorticosterone equivalent per minute.

It is not known whether 11-oxycortioids antagonise the sodium retaining activity of DOCA in Spencer's method as it does in the author's. Such antagonism does not occur in Simpson and Tait's method, the big difference between their results and the author's is to be expected.

Bibile (1953) calculated Vogt's (1943) cold test estimation of the dog adrenal gland output of cortisone to be 18 µg. per minute. Using the eosinophil test, Bibile estimated this output to be 11.3 µg. cortisone per gram per minute. The eosinophil test is to a great extent, a specific test for 11-oxycorticoids.

From the author's study of the neutralising power of hydrocortisone (Compound F) on the sodium retaining effect of DOCA, it follows that 4.0 µg. of DOCA were not enough to neutralise 10.0 µg. of hydrocortisone. Since, according to Bush, hydrocortisone is the main corticoid produced by the dog's adrenal, it is possible to estimate from Bibile's figure that the mineralo-cortical activity in the dog adrenal venous plasma in the author's experiments was probably greater than 5.0 µg. DOCA.

Vogt/

Vogt (1944) reported that within reasonable limits, changes in blood pressure and blood flow had no effect on the rate of cortical secretion. In the one experiment carried out on a dog which seemed to have some salt-retaining activity in its adrenal venous blood, the blood pressure during the collection of the samples was twice as high as the blood pressure of the other dogs in which no salt-retaining activity was demonstrable, or very little.

It is not possible to decide whether these differences are due to variations in the amount of mineralo-corticoids or in the quantity of hydro-cortisone secreted. Bush (1953) reported that in the rabbit, the adrenal cortex secretes Compounds F. and B. almost exclusively. The $\frac{F}{B}$ ratio being 0.1. This is in contrast to the dog which secretes mainly Compound F.

In the reported work here, Compound B. had the least potency in antagonising the sodium-retaining effect of DOCA. 10 µg. Compound B. being not enough to neutralise 4.0 µg. DOCA. From Bush's figures it is calculated that the average concentration in the rabbit is 3.2 µg. Compound B. per ml. of adrenal plasma.

The rabbit thus seemed to be an animal where the/

the neutralising effect of 11-oxycorticoids against the sodium-retaining action of DOCA-like substances, would cause the least interference.

The mineralo-cortical activity of the adrenal venous plasma in a rabbit was indeed found to be highly significant ($P < 0.001$) and of the order of 2.7 µg. DOCA equivalent per gram adrenal gland per minute.

SUMMARY.

A qualitative and a quantitative method of estimating mineralo-cortical activities are reported. Between them they can supply a simple routine method of assay. The quantitative method has a reasonable range of sensitivity but the degree of accuracy attained is low. Some of the factors involved in this kind of test are investigated.

A comparative study of sodium-retaining activity of some pure corticoids indicated the presence of a qualitative difference between 11-oxycorticoids and DOCA. Antagonism between DOCA and some of these corticoids is demonstrated and quantitative data on this antagonism are reported.

Attempts/

Attempts at measuring the mineralo-cortical activity of adrenal venous plasma in the dog and the rabbit are reported. The maximum figures obtained were:

In the dog, 0.9 µg. DOCA equivalent per gram adrenal gland per minute.

In the rabbit, 2.7 µg. DOCA equivalent per gram adrenal gland per minute.

Attention is drawn to the significance of these figures in relation to the reported antagonism between the different steroids.

The significance of the qualitative differences between the actions of different corticoids is discussed. The role played by the conditions of the experiment and the type of response chosen is pointed out.

It is suggested that the different steroids have a complementary relationship towards each other when their overall functions are considered, their antagonism or parallelism being incidental and dependent on the overall conditions of the observations.

Glycyrrhetinic Acid

Introduction

Several reports have attributed a DOCA-like activity to liquorice extract as well as to "glycyrrhetinic acid" one of its components.

From the therapeutic point of view, there seems to be little place left for a substance having such an activity. From the pharmacological point of view however, it would be of great interest if a non-steroid compound like glycyrrhetinic acid is shown to possess one of the main activities of the cortical-steroids.

All the reported results on liquorice activity in this connection have been carried out on man. None of these results have been successfully reproduced in laboratory animals.

It was hoped that a study of the action of glycyrrhetinic acid on water and electrolyte metabolism in conjunction with the previously reported study of adrenal corticoids might furnish a promising approach to the problem of mineralo-cortical activity.

Review of Literature

Revers (1948) treated a series of peptic ulcer patients with succus liquiritiae and reported that one out of five of these patients developed oedema. The oedema started in the face and later spread to other parts of the body. Headache, shortness of breath on exertion and pain in the upper abdomen accompanied the oedema and were relieved with it when the dose of the succus was reduced. In some patients, the treatment had to be abandoned altogether because of the severity of the symptoms. On readministering the drug, the same picture was reproduced.

Van den Berghe and Pannekoek confirmed these observations (1950).

Molhuysen et al (1950), giving patients from 20 - 45 g of the succus daily in 8 equal doses and collecting the urine every three hours, found the 3rd and 4th specimens smaller than control specimens. They concluded that the effect of the drug was to stimulate the renal tubules to reabsorb water and chlorides. They suggested that sodium is affected in the same way. They were able to reproduce the same/

same kind of results with an extract consisting mainly of glycyrrhizin. Ammonium glycyrrhizate also was found to be effective. They concluded that the active principle must be either glycyrrhizin or another substance precipitated with it by acid.

Comparing the effect of succus liquiritiae with that of deoxycortone, they found that 20 g of the succus gave greater retention of sodium chloride and water than that caused by two daily injections of 10 mg of deoxycortone. The plasma volume had increased and so had the cardiac output. Potassium balance became markedly negative while sodium balance was strongly positive. The potassium content of the plasma was decreased below the control level. They pointed out that an increase in cardiac output is usually followed by increased output of the three components of the extracellular fluid (Na, Cl. and water). As a result of the antagonistic mechanism long continued treatment with either liquorice or deoxycortone must be self limiting.

On withdrawing either succus liquiritiae or deoxycortone there was a rebound and this was more pronounced/

pronounced and quicker with deoxycortone.

They failed to produce similar results on treating a patient with Addison's disease, and concluded the drug acts through the adrenals.

Groen et al (1951) confirmed Borst's findings on non-Addisonian persons. They reported liquorice to be effective in treating Addison's disease. It had a slower and more prolonged action than DOCA and was effective by the mouth.

The sodium balance in Addisonian patients became positive when they were treated with either liquorice or DOCA. The potassium balance reached equilibrium under DOCA treatment and became markedly negative with liquorice.

Groen et al (1952) found glycyrrhizinic acid to possess a DOCA-like effect. Its effect on sodium and chloride in an Addisonian patient was prompt, while its effect on potassium had a lag of one day. Sodium and chloride balance was maintained in a positive state with either the ammonium or potassium salt of the acid while the potassium balance became negative. On withdrawing the drug , sodium and chloride balance became negative while the potassium balance became/

became positive. A crude extract of liquorice was able to reverse this deterioration effectively and to maintain the improvement.

Combining their observation with Borst's that a liquorice extract from which glycyrrhizinic acid has been precipitated lost its activity, the authors concluded that glycyrrhizinic acid is the active principle in liquorice. They draw attention to certain features of similarity between the structure of glycyrrhetinic acid (one component of glycyrrhezinic acid) and corticosterone.

Card et al (1953) regulated the food intake of normal subjects so that it contained 9 g. of sodium daily, and reported a gain in weight to be the most striking effect of liquorice treatment. This was accompanied by positive fluid balance, and a fall in chloride excretion in urine. DOCA caused a positive fluid balance in one subject only and this was smaller than that due to liquorice. Maximum gain in weight was achieved on the 5-9th day and the positive fluid balance reached its maximum during this period. No further gain in weight was observed in subsequent periods even though the dose of liquorice was doubled in one subject. Two-thirds of the gain in weight was accounted for by an increase of the extracellular space (thiocyanate space).

Changes/

Changes in sodium and potassium balances were not marked. The interesting feature was that changes in sodium balance followed the changes in fluid balance and weight. A small fall in the urinary $\frac{Na}{K}$ ratio was noted during liquorice treatment. This fall was larger than that observed with DOCA treatment in the same subjects. When different fractions of the liquorice extract were screened on normal subjects while sodium intake was not controlled, no definite evidence of activity was detected. The authors explained this by the considerable variation in response from one subject to another.

Glycyrrhetinic acid, in doses of 0.5 g daily, exerted a water, sodium and chloride effect but the influence on potassium balance was not significant.

In an Addison's disease patient, 50 mg q.i.d. caused an increase in weight and a fall in urinary volume with indications of blood dilution. The $\frac{Na}{K}$ ratio in the serum increased.

Borst et al (1953) treated two Addisonian patients with liquorice extract and reported that in one of them they failed to observe any effect. In the/

the other, liquorice had an effect but its ability to accentuate the effect of small doses of cortisone was more marked. On repetition, in the 2nd patient the effect of liquorice by itself was not reproduced, while its accentuating action on cortisone was. The authors thought it possible that liquorice may be potentiated by the endogenous corticoids. To investigate this possibility, they treated a Simmonds-Sheehan patient, and found liquorice very effective in retaining water and sodium, while potassium was not retained. This beneficial effect was reproduced with a combination of smaller doses of liquorice supplemented by cortisone as well as with liquorice and ACTH. The last combination failed to affect an Addisonian patient.

Pelser et al (1953) reported that pure glycyrrhizinic acid produced retention of sodium and chloride and increased potassium excretion in a patient with Addison's disease. Glycyrrhetinic acid in doses of 3-5 g orally per day corrected the abnormal electrolyte balances in two similar patients. Chemically pure preparations of these two acids had similar effects. Glycyrrhetinic acid given intramuscularly in doses of 60 - 100 mg seemed to have a similar action/

action but was found to be an irritant. Wide individual variations were noted in the reaction of four different patients to the same dose of liquorice extract. The maintenance doses necessary in the four cases were found to be 3, 20, 30 and 40 g of liquorice per day. The DOCA-like effect of liquorice, though found in man, has not been demonstrable in animals.

Nelemans-Stamperius (1949) found no effect on water or mineral metabolism in animals.

Card et al (1953) carried out a series of experiments on rats and guinea-pigs to investigate the actions of liquorice on water and electrolyte metabolism. Normal rats were given a dose of liquorice equivalent to that effective in man. At the same time, they were given a saline drink. No increase in weight was observed even when the dose of liquorice was increased to the extent of the equivalent of 180 g per day to a 60 kgm man. Liquorice failed to prolong the survival time of adrenalectomised rats.

Glycyrrhetinic acid given orally or subcutaneously to normal rats caused no increase in weight. When given/

given to adrenalectomised rats by both routes no increase in weight or survival time was achieved even when ten times the equivalent dose used in man was administered.

In normal guinea-pigs subcutaneous glycyrrhetinic acid caused no increase in weight in ten times the equivalent human doses.

Some effects of liquorice extract on isolated organs has been reported by Bijlsma-Nelemans et al (1948). According to these authors, liquorice extract has a decided spasmolytic action. On stimulating the isolated intestines of rats, guinea-pig, pig or rabbit with minimal doses of acetylcholine, histamine or barium chloride respectively, they found that 50 mg of liquorice extract added to 60 ml of liquid was the smallest concentration necessary for definite spasmolytic action. By employing atropine to antagonise the action of acetylcholine, benadryl against histamine and papaverine against acetylcholine, histamine and barium chloride, they found liquorice to have a papaverine-like action.

Glycyrrhetinic Acid

The crude extract of liquorice contains about 5 per cent of its weight as glycyrrhizin, the potassium and calcium salts of glycyrrhizinic acid. (The B.P.C. 1949 gives the percentage as 2-5 per cent while the U.S. Dispensary gives it as 5-10 per cent).

Glycyrrhizinic acid is a conjugate of glycuronic acid and glycyrrhetinic acid.

Glycyrrhetinic acid is a white powder freely soluble in alcohol, but it is precipitated on dilution with water. It can form a fine suspension if the alcohol is gradually diluted to a concentration not below 20 per cent. The acid is soluble in propylene glycol on heating.

Effect on Electrolyte Balance: Urine.

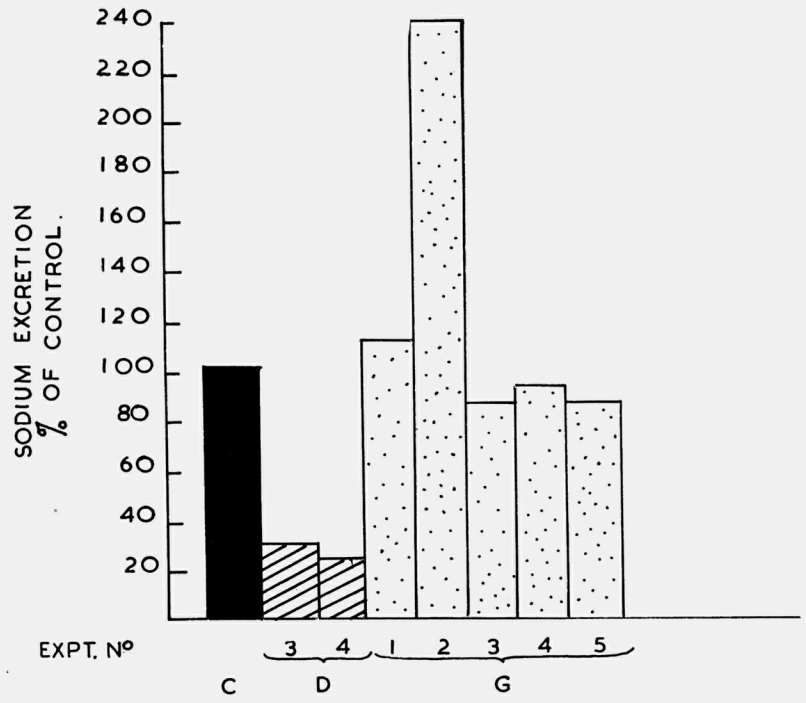
Method.

The method previously described for assaying mineralo-cortical activity was employed.

The test groups of rats were injected with 0.1 ml. propylene glycol subcutaneously each containing 200-400 µg. of the glycyrrhetinic acid.

The/

Figure 8.



Effect of glycyrrhetinic acid on sodium excretion in adrenalectomised rats.

C = Control

D = DOCA

G = Glycyrrhetinic

(TABLE XIV)

The period of collecting urine was cut short to 4 hours instead of 6 hours in two experiments. It was possible with this shorter period to study the effect of the antidiuretic action of the acid on sodium excretion. In experiments on diuresis reported later, the antidiuretic effect of the acid seemed to wear off in 4 hours.

Results - Urinary sodium excretion.

In glycyrrhetinic acid treated rats (Table XIV and Fig. 8) sodium excretion was not inhibited or altered to any significant degree if the collection of urine was continued for a period sufficiently long to allow urine to flow. With doses of 0.2 and 0.4 mg. glycyrrhetinic acid a 6 hours' collection was sufficient for this purpose. When 1.0 µg DOCA per rat was administered under the same conditions significant sodium retention was achieved ($P = .03$).

When urine was collected over 4 hours only, and doses of 0.4 mg. of the acid were administered per rat the mean sodium excretion for the whole group was appreciably below that of the control group. Under these conditions, however, it was observed,

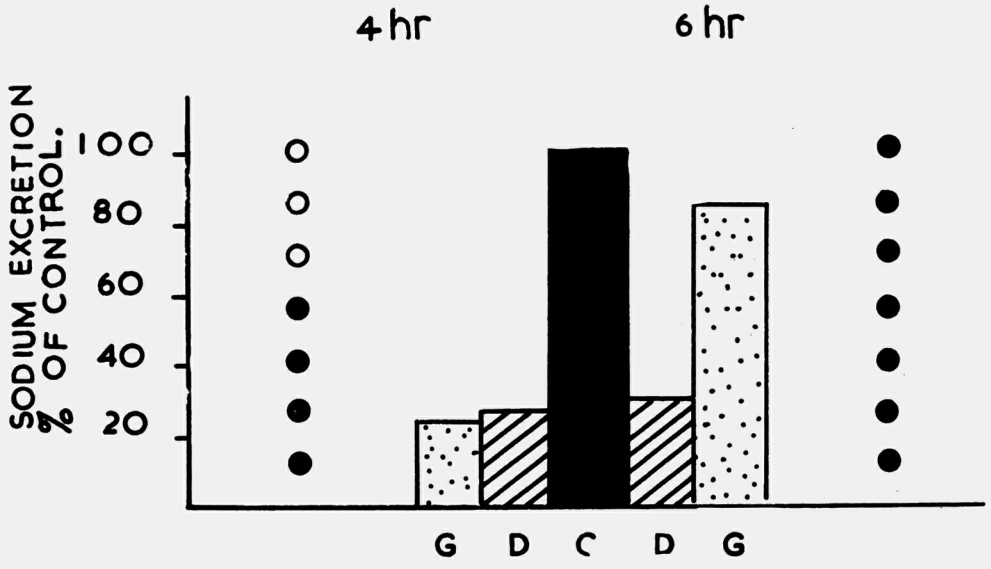
TABLE XIV

Effect of Glycyrrhetinic Acid on Urinary Sodium Excretion.

No. of expt	Time of collecting urine hr	Propylene glycol		Glycyrrhetinic Acid and DOCA			
		No. of animals	Mean Na excretion mg	No. of animals	Dose µg	Mean Na excretion mg	Na excretion % of control
1	6	7	3.45	8	400 glyc.	3.78	109.6%
2	6	6	0.61	6	200 glyc.	1.45	240%
3	6	7	2.66	7	200 glyc.	2.25	84.6%
				7	1.0 DOCA	0.899	29.6%
4	6	5	2.53	6	400 glyc.	2.23	89.2%
				5	5.0 DOCA	0.573	22.6%
5	6	6	1.58	6	400 glyc.	1.29	84.3%
6	4	7	1.48	4 and 3[1]	400 glyc.	0.56	37.8%
7	4	7	1.66	4 and 3[1]	400 glyc.	0.39	23.5%
				7	1.5 DOCA	0.43	26.0%

[1] Number of animals which excreted no urine at all. They are included in the calculation for mean Na excretion for the whole group.

Figure 8a



Effect of glycyrrhetinic acid (4 hr and 6 hr after administration) on diuresis and sodium excretion.

O = Rat excreting no urine

● = Rat excreting urine

C = Control

D = DOCA

G = Glycyrrhetinic

(TABLE XIV)

observed that a number of the glycyrrhetinic-acid-treated rats did not excrete any urine at all and thus did not contribute to the overall excretion of sodium by the group. With a 4 hours' collection 1.5 µg DOCA caused a significant sodium retention ($P = .01$) while the volume of urine collected from any rat was not appreciably smaller than that collected in a 6 hours' collection (Fig.8a).

Urinary potassium excretion.

In two out of the five experiments of Table XIV the relative potassium excretion was enhanced by glycyrrhetinic acid to a greater extent than that caused by DOCA. One example is given in Table XV.

These increases, however, are not significant and were not always observed. The load solution administered in this method of assay contains a minute amount of potassium (0.2 mg) and so most of the potassium excreted must come from that of the body fluids. The animals were not in an advanced state of adrenal deficiency and as such were not expected/

Expt. 4 Table XIV

TABLE XV

Potassium Excretion.

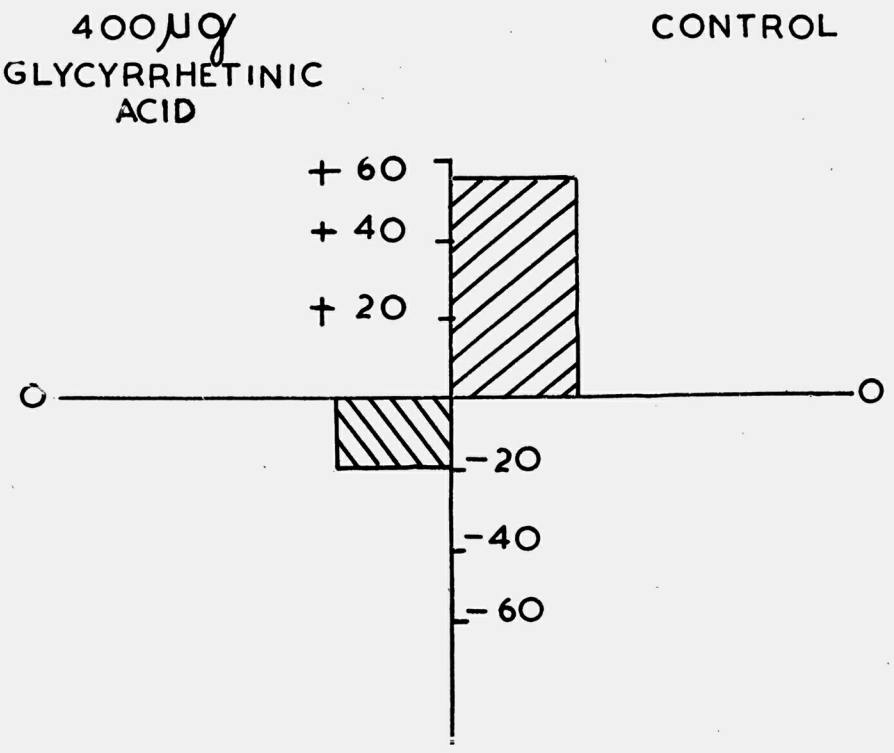
Control Group			DOCA 5mg			Glycyrrhetinic 400mg		
Mean Na excretion (mg)	Mean K excretion (mg)	$\frac{K}{Na}$ x100	Mean Na excretion (mg)	Mean K excretion (mg)	$\frac{K}{Na}$ x100	Mean Na excretion (mg)	Mean K excretion (mg)	$\frac{K}{Na}$ x 100
2.53	1.06	42	0.573	0.47	82	2.23	2.02	90.7

TABLE XVI

Potassium Excretion

Control				Glycyrrhetinic 400mg			
No. of rats	Mean Na excretion mg	Mean K excretion mg	$\frac{K}{Na}$ x100	No, of rats	Mean Na excretion mg	Mean K excretion mg	$\frac{K}{Na}$ x100
6	1.05	0.86	81.9	6	1.62	2.41	148.8

Figure 9.



Effect of glycyrrhetinic acid on potassium balance.

Control = adrenalectomised rats having a highly positive balance. This is reversed to a negative balance in glycyrrhetinic treated rats (400 µg).

Each rat received 3.8 mg potassium chloride, subcutaneously

expected to have a marked degree of potassium retention. To simulate this state of affairs the assay was carried out with one important change. The second load of the assay was replaced by a potassium chloride solution containing 2.0 mg potassium in 2.0 ml distilled water (i.e. 3.8 mg potassium chloride). The urine collection was carried over a period of 6 hours and the potassium excretion was found to be significantly increased by the glycyrrhetinic acid in 0.4 mg doses ($P < .02$), Table XVI,(Figure 9).

Blood electrolytes.

Method.

300 g. male albino rats were adrenalectomised under ether anaesthesia and kept on rat cubes and 1 per cent sodium chloride-5 per cent glucose drinking solution for 4 days. On the 5th day, the animals were divided into three equal groups and weighed. Cubes and water were continued. Injections were started and continued daily for 5 days. No saline drink was administered during this period.

Groups I and IV received subcutaneous injections of/

of 0.25 ml propylene glycol per rat.

Group II received 3 mg glycyrrhetinic acid dissolved in 0.25 ml propylene glycol.

Group III received 1 mg of DOCA in 0.25 ml propylene glycol.

Group IV were non-adrenalectomised rats from the same colony and of the same weight. Each rat received 0.25 ml of propylene glycol subcutaneously.

On the 5th day, 4 hours after the injection, the animals were weighed again, anaesthetised with an intraperitoneal injection of pentobarbital sodium 6% solution, the dose administered was 0.7 ml per kg body weight. The rats went quickly under anaesthesia. The chest was opened and the heart punctured with sharp scissors. The first few drops of blood were discarded and the flow was then collected into a centrifuge tube. The blood was allowed to clot, was later centrifuged and the serum pipetted off. After dilution with distilled water, the sodium and potassium contents were estimated with the flame photometer.

Results.

Table XVII and Fig. 10 illustrate the findings of this experiment. DOCA in 1.0 mg doses per day successfully/

successfully maintained the serum sodium of adrenalectomised rats above the normal level for the non-adrenalectomised rats of the same colony ($P < 0.01$). The serum sodium of the DOCA treated adrenalectomised rats was significantly elevated above that of the adrenalectomised propylene glycol injected controls, ($P < 0.001$). These controls showed the classical picture of adrenal deficiency, their serum sodium dropped below the normal level ($P < 0.001$) and their serum potassium rose ($P < 0.001$). The serum potassium of the DOCA treated group showed a slight drop, but was here not significantly higher than the level of the normal controls ($P = .1-.2$).

Glycyrrhetinic acid did not cause a significant rise of serum sodium or drop of serum potassium. The dose administered was 3.0 mg per 300 g rat per day, this is equivalent to 600 mg glycyrrhetinic acid to a 60 kg man per day. A dose well within the range reported to be effective in man.

DOCA successfully maintained all the animals in the group alive and in good shape. The glycyrrhetinic acid group started to show weakness much earlier even than the adrenalectomised controls.

The/

TABLE XVII.

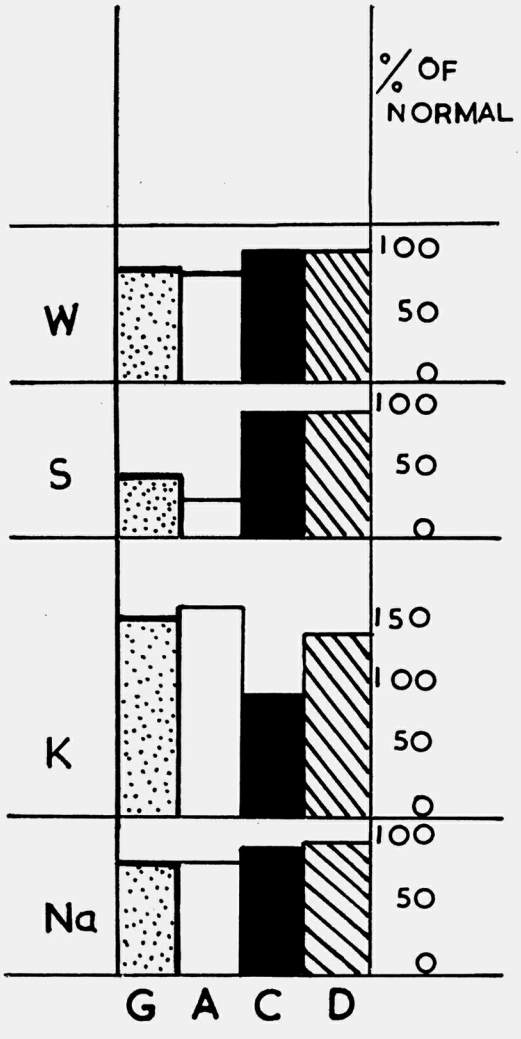
Effects of Glycyrrhetinic Acid on Serum Electrolytes in Rats.

Adrenalectomised rats[1]

Group	No.of rats per group	Mean serum Na % of normal	Significance of Na change	Mean serum K % of normal	Significance of K change	Mean gain in weight g.	Survival %
Normal 0.25ml (prop. gly.)	8	100	-	100	-	+ 0.1	100
Non-treated.	10	88.7	from normal $P < 0.001$	166.7	from normal P=.01 -.02	- 10.5	30
1 mg DOCA per day per rat	8	105.9	from adrenalectomised non-treated $P < 0.001$ from normal $P < 0.01$	144.7	from adrenalectomised non-treated not significant from normal not significant	+ 0.5	100
3mg glycy. per day per rat	10	91.3	from adrenalectomised non-treated not significant from normal $P > 0.001$	163.5	from adrenalectomised non-treated not significant from normal $P = .02$	- 8.6	50

[1] Each rat received 0.25 ml propylene glycol subcutaneously.

Figure 10



Effect of glycyrrhetinic acid treatment on serum electrolytes, survival and weight of adrenalectomised rats.

Na = Mean serum sodium
K = Mean serum potassium
S = Mean survival
W = Mean weight
C = intact rats (non-treated)
A = adrenalectomised rats (non-treated)
D = adrenalectomised 1 mg DOCA per rat per day.
G = adrenalectomised 3 mg Glycy. " " " "

(TABLE XVII)

The survival of the group however, was slightly, but not significantly, better than the controls, (Fig. 10).

As regards weight, the groups of adrenalectomised animals, either treated with glycyrrhetinic acid or left without treatment as controls, showed a marked loss of weight while non-adrenalectomised animals and DOCA treated animals showed no weight loss.

Antidiuretic Action

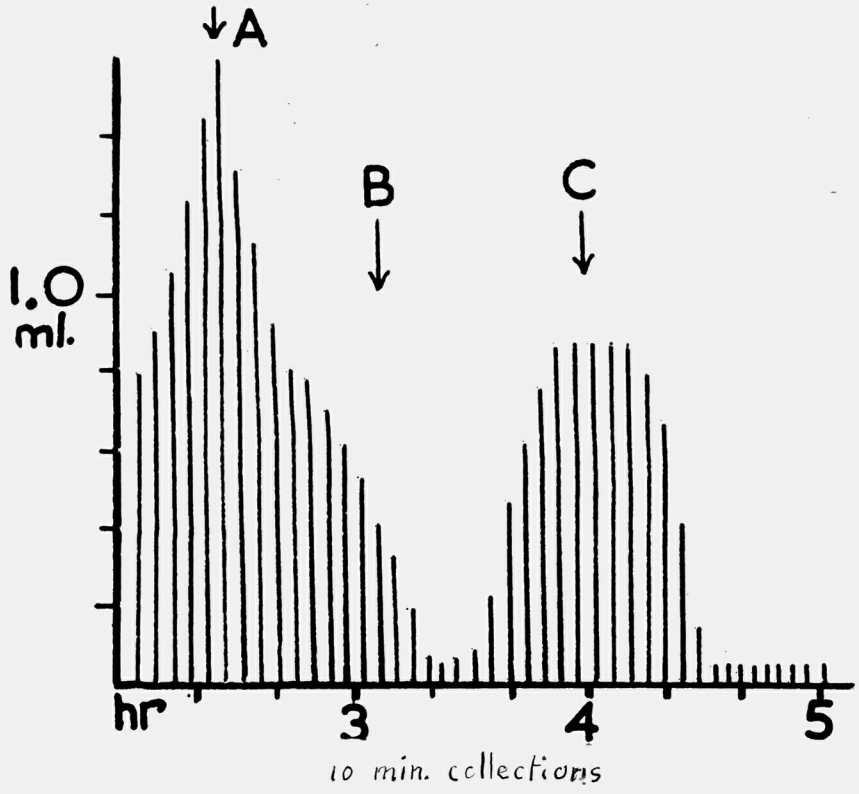
Rat

Method.

Male rats above 250 g were used. The rats were starved overnight leaving them free access to tap water. About 10 a.m. anaesthesia was administered in the form of 10 per cent alcohol in tap water; 5.0ml/ 100 gm. body weight of the solution was administered by a thin rubber catheter into the stomach and the rat was kept in a warm place. In about 30 minutes, the effect of the alcohol started to show. If unconsciousness was not achieved in 45 minutes, another dose was given (1.5 ml of the same solution/ 100 g body weight). A small suprapubic cut was made, the bladder secured and a thin polythene tube (3 mm internal/

internal diameter) was introduced into the bladder through the apex and tied in securely. The penis was tied up to prevent any urine escaping except through the polythene tube. If the anaesthesia was not deep enough to perform the operation a whiff of ether was used. In experiments where the drug was intended to be introduced orally, this was all that was required. Otherwise, if the drug was to be introduced intravenously, a thin polythene cannula was introduced in the jugular vein. For the first hour or two, no significant diuresis was observed for a period depending on the degree of shock resulting from the operation. In the meantime, a fine thermometer was introduced per rectum and the animals placed on a box warmed by electric bulbs and their temperature kept as constant as possible at 37° by switching on and off the bulbs. The end of the catheter was fixed to a graduated centrifuge tube and a reading of the urine collected was recorded every 10 minutes. When diuresis started, a new load was introduced by stomach tube of 10.0-14.0 ml of water, or 3 per cent alcohol in tap water if anaesthesia was getting light. The degree of anaesthesia was kept deep enough to prevent the animals from moving or responding/

Figure 11.



Effect of glycyrrhetinic acid on diuresis in rat.

Oral administration.

A = 1 mg glycy. in 3 ml 10% ethyl alcohol.

B = 10 ml water

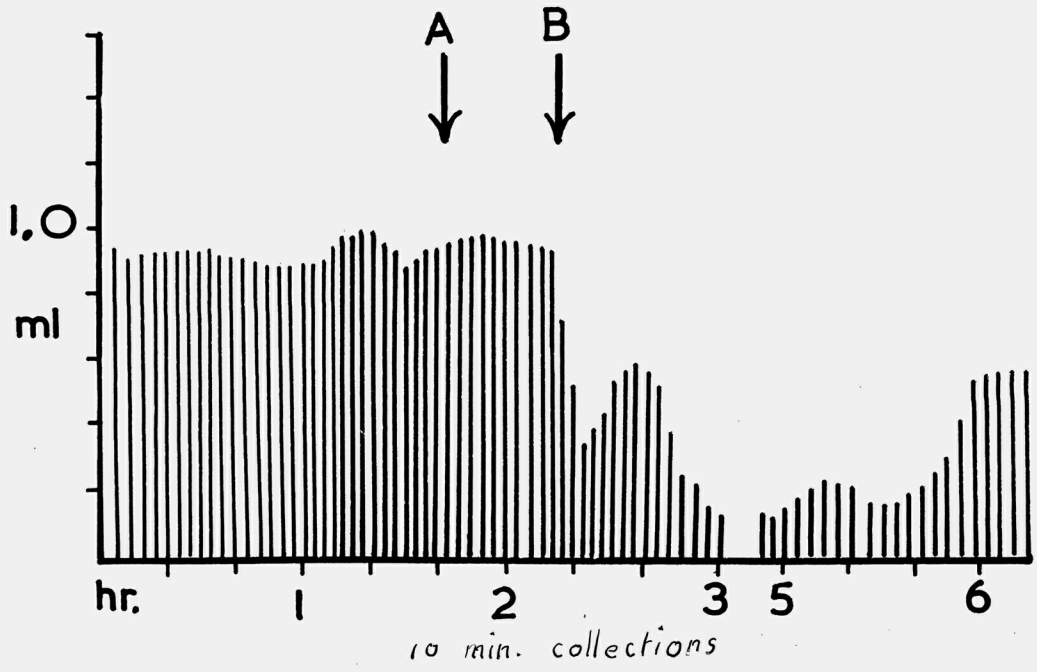
C = 1 mg glycy. in 3 ml 10% ethyl alcohol.

responding to external mechanical stimuli, but not any deeper otherwise diuresis was inhibited. Whenever 8 ml of urine was collected they were replaced by 10 ml of water by stomach tube or 3 per cent alcohol, if required. The solvent in which the drug was administered was administered in the same manner as the drug to test its effect on diuresis. When introducing the drug orally it was given as a fine suspension in 10 per cent alcohol, 1-3 ml. When the drug was introduced intravenously it was given as a clear solution in propylene glycol (0.05 - 0.2 ml) and washed down the cannula with the minimum amount of saline possible. Care was taken to cut down any amount of handling, noise or change of temperature as all these factors affected the flow of diuresis in an unpredictable manner.

Results.

Glycyrrhetinic acid, whether introduced by the mouth or intravenously, caused an appreciable suppression of the rate of diuresis. If given in doses in the range of 1 mg intravenously to a 300 g rat it caused almost complete suppression of urine from which the recovery was very slow, taking about 3-4 hours. The response, however, was not uniform in/

Figure 12.



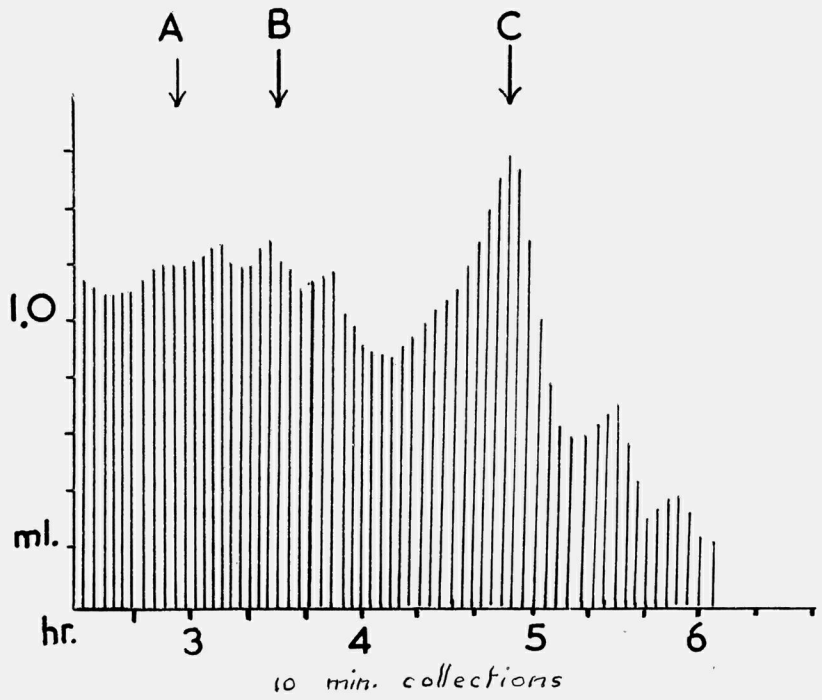
Effect of glycyrrhetinic acid on diuresis in rat.

Intravenous administration.

A = 0.1 ml propylene glycol washed in with 0.6 ml saline.

B = Same as A, but containing 100 µg glycyrrhetinic acid.

Figure 13.



Effect of glycyrrhetinic acid on diuresis in rat

Intravenously.

A = .05 ml propylene glycol washed in with .1 saline.

B = same as A, but containing 40 µg glycyrrhetinic acid.

C = same as A, but containing 150 µg glycyrrhetinic acid.

in different rats. Some rats responded to a small dose of 200 µg more markedly than to bigger doses in other rats.

As shown in the figures (E.g. 11) 1 mg orally, almost suppressed diuresis in 350 g rats.

When given intravenously (Fig.12) 0.1 ml propylene glycol alone had no effect, while if the same fluid contained 100 µg of the acid the diuresis diminished appreciably for 4 hours and then did not regain the initial level which however was maintained more or less constant for two hours before the drug was introduced.

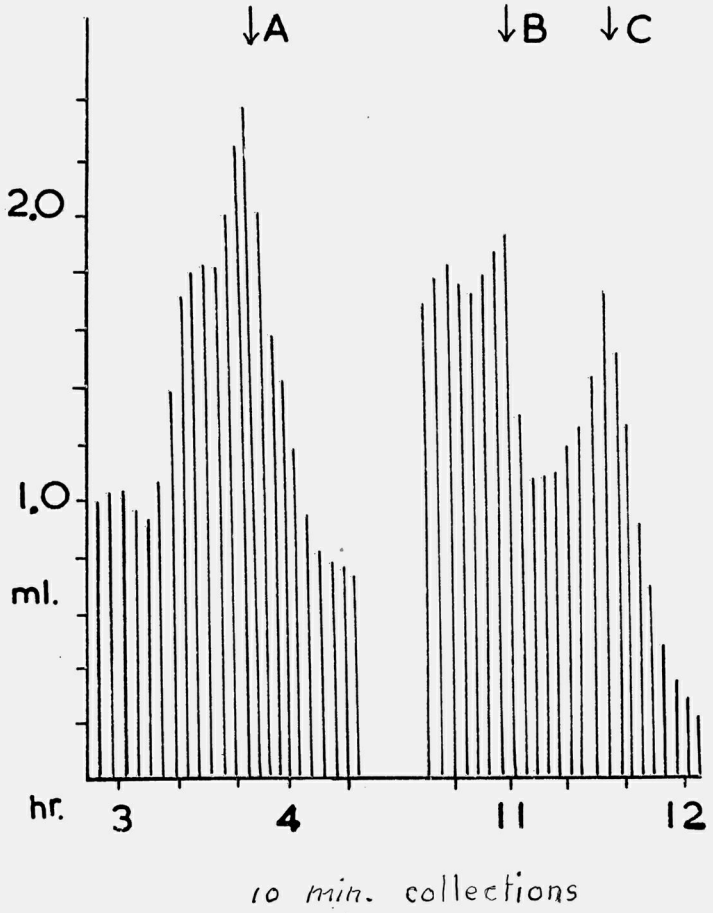
The least effective dose for a 280 g rat was found to be around 40 µg (Fig.13) while 150 µg had a marked effect on this rat.

On the other hand, while 40 µg was effective on a 360 g rat after 4 hours from the operation, after 11 hours 0.5 mg had a smaller effect but 1.0 mg was able to suppress diuresis. (Fig.14).

When injecting the drug intravenously, a slight haemorragic tinge in the urine was noticed. This tinge however, was noticed with propylene glycol alone on a few occasions.

When the drug was injected intravenously, it had to/

Figure 14.



Effect of glycyrrhetinic acid on diuresis

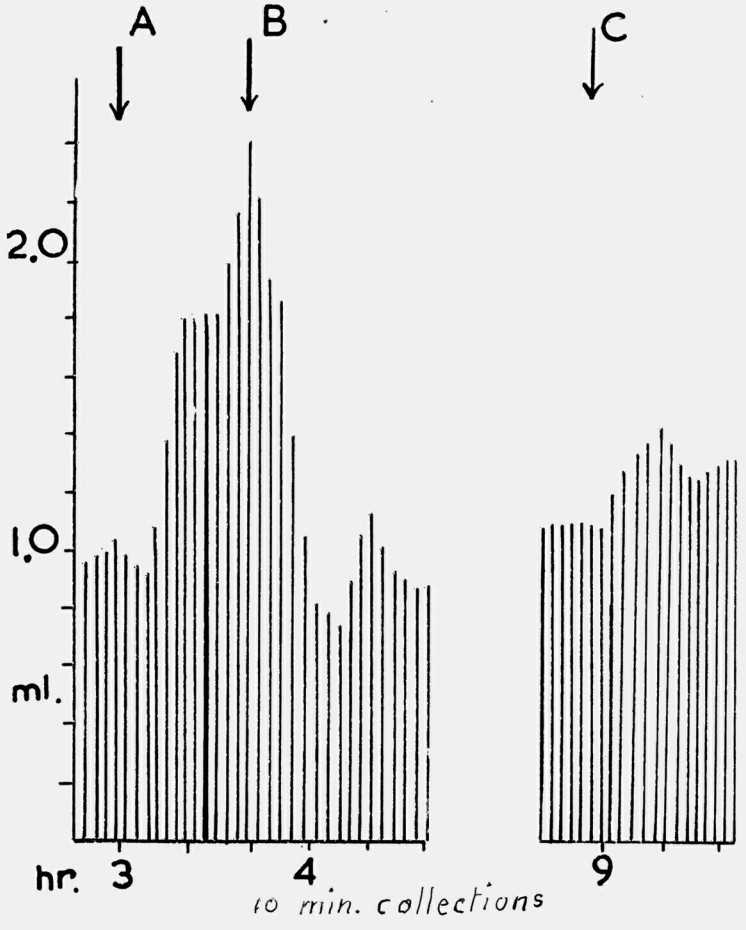
Intravenously.

A = 40 µg glycy. in .1 propylene glycol washed in with 0.5 saline.

B = 500 µg glycy. in same as A.

C = 1000 µg glycy. in same as A.

Figure 15.



Effect of glycyrrhetinic acid on diuresis in rats.
Intravenously

A = 0.1 ml propylene glycol washed in with 0.5 ml saline (injected rapidly).

B = same as A, but containing 40 µg glycy. acid.

C = same as A, but injected slowly.

to be given very slowly, otherwise propylene glycol by itself could cause a diuresis which was found to mask the effect of the glycyrrhetinic acid. This may be the explanation why sometimes, higher doses failed to give bigger responses (Fig.15).

Antidiuretic Action

Dog.

Method.

A collie bitch of 10 kg body weight was used. A perineotomy operation was performed on the bitch under ether anaesthesia to facilitate the introduction of the urinary catheter into the bladder.

A period of three weeks training was necessary thereafter, before the full co-operation of the dog was gained.

The night before the experiment, the dog was kept fasting with free access to water. At 9 a.m. the next morning, a stomach tube was introduced and 70 ml of tap water given in three portions. When glycyrrhetinic acid was to be given 350 mg were suspended in the first portion and washed down by the last two. The dog was then allowed free movement with/

with no access to food and water for 2 hours. At 11 a.m. a self-retaining catheter was well sterilised and introduced into the urinary bladder. The urine collected was discarded. The dog was tied up to a Pavlov stand and the suspending cords were adjusted to allow the dog to stand comfortably on its feet. At 11.15 a.m. 250 ml of tap water was introduced by a stomach tube and the self-retaining catheter connected to a collecting tube pouring into a measuring cylinder. Diuresis ensued in 20-25 minutes and gradually increased. At 11.55 a.m. 300 ml of tap water was again introduced by stomach tube. From 12 noon, urine was collected at 5 minute intervals for a period of 1 hour and 40 minutes. The overall urine collection was continued for 3 hours and 50 minutes since the introduction of the first water load at 11.15 a.m. The time of this first load was taken as zero hour. Throughout the experiment, nothing was forced on the dog and all external interferences were cut down as far as possible. A constant temperature and complete quietness were essential.

In the second set of experiments, the glycyrrhetinic acid was introduced as a fine suspension in

10/

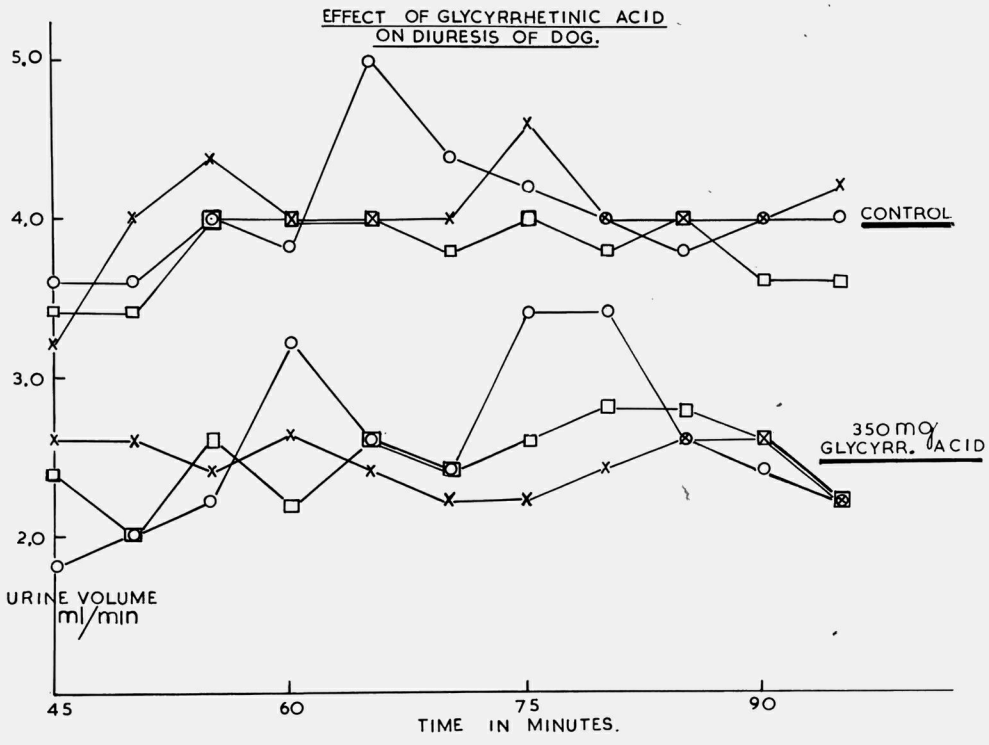
TABLE XVIII.

Effect of Glycyrrhetinic Acid on Diuresis in Dog.

(5 minutes collections of urine)

No. of Expt	Amount of glycy. acid or DOCA	Suspending medium.	Urine excretion in ml/minute											Total urine collection in 3hrs 50mi in ml.
			1	2	3	4	5	6	7	8	9	10	11	
1	-	70 ml water	3.6	3.6	4	3.8	5	4.4	4.2	4	3.8	4	4	490
2	-	70 ml water	3.4	3.4	4	4	4	3.8	4	3.8	4	3.6	3.6	485
3	-	70 ml water	3.2	4	4.4	4	4	4	4.6	4	4	4	4.2	475
4	350mg glycy.	70 ml water	1.8	2	2.2	3.2	2.6	2.4	3.4	3.4	2.6	2.4	2.2	372
5	350mg glycy.	70 ml water	2.6	2.6	2.4	2.6	2.4	2.2	2.2	2.4	2.6	2.6	2.2	388
6	350mg glycy.	70 ml water	2.4	2	2.6	2.2	2.6	2.4	2.6	2.8	2.8	2.6	2.2	405
7	-	70 ml 10% alcohol	4.4	4.6	5.6	5.4	5.2	5.4	4.8	4.4	4.2	4.4	4.6	515
8	350mg glycy.	70 ml 10% alcohol	2.8	3.2	3.4	3.6	3.8	3.8	3.6	3.4	3.2	3.2	2.8	457
9	2.5mg DOCA s.c.	70 ml water	3	3	3.4	3.4	4	3.6	4.4	4	4	3.2	3.2	532

Figure 16.



Five minutes urine collections after two water loads of 250 and 300 ml respectively.

Three control and three test experiments on the same dog.

(TABLE XVIII)

10 per cent alcohol. The volume of the suspension vehicle being again 70 ml divided into three portions.

In one experiment in which the dog received no glycyrrhetinic acid in the 70 ml of water, 2.5 mg DOCA were injected subcutaneously at 9 a.m. and the rest of the experiment continued as before.

Results. (Table XVIII and Fig.16)

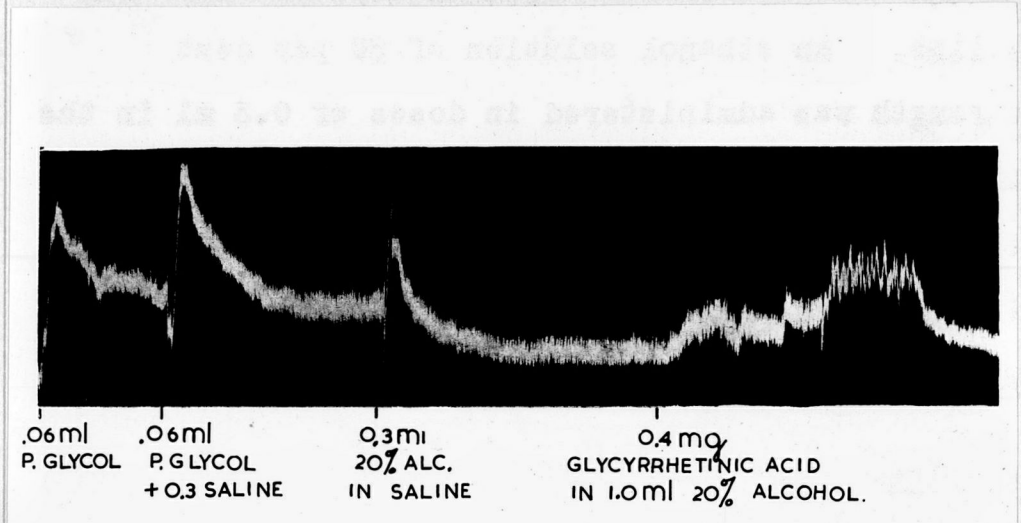
In control experiments with no glycyrrhetinic acid, a high rate of diuresis was observed. This rate seemed to be a little higher when 70 ml of 10 per cent alcohol were used instead of water. In both cases, glycyrrhetinic acid seemed to cause a depression of the rate of diuresis. This was reflected in the rate of diuresis as well as in the volume of urine excreted over a period of 3 hours and 50 minutes. In the one experiment carried out on DOCA no such effect was observed.

Blood pressure.

Method.

A 200 g rat under urethane anaesthesia, with the femoral vein cannulated and connected to a saline-filled U-shaped pipette and the carotid artery cannulated and connected to a sensitive blood-pressure/

Figure 17.



Effect of glycyrrhetinic acid on the rat's blood pressure.

pressure manometer, was used.

Propylene glycol .06 ml were administered through the femoral vein and washed down with 0.3 ml saline. An ethanol solution of 20 per cent strength was administered in doses of 0.3 ml in the same way. 1.0 ml of the alcoholic solution containing 0.4 mg glycyrrhetinic acid was then administered and washed down with saline. This administration was carried out at a very slow rate.

Results.

Propylene glycol did not cause any depression of the blood pressure. It caused a rise which was not maintained and not smaller than that produced by the same volume of ethanol in saline, (Fig. 17). Glycyrrhetinic acid also had no hypotensive effect in the dose used.

Water Retaining Effect of Glycyrrhetinic Acid Site of Action

The purpose of these experiments was:

1. To confirm the antidiuretic action of the drug in non-anaesthetised rats.
2. To investigate whether an intact posterior lobe of the pituitary is essential for the drug to exert its anti-diuretic effect.
3. To locate the site of action.

1. Method.

Albino rats around 150 g.weight were used. Food was withdrawn at 5.0 p.m. the previous night; water was withdrawn at 9.0 a.m. on the day of the experiment. The animals were divided into two equal groups, one to act as control and the other as test. At 11.0 a.m. each control rat was given 1.0 ml. of propylene glycol by stomach tube. The test group had 100 mg of glycyrrhetinic acid dissolved in 1.0 ml. of propylene glycol. The rats were kept in a quiet place for three hours. At 2.0 p.m./

2.0 p.m. each animal got 12 ml of tap water by stomach tube; its bladder was emptied by gentle pulling of the tail and the rat placed in a metabolic cage for collecting urine. The cage consisted of a waxed tin funnel covered by a waxed wire mesh and surmounted by a cylinder of wire mesh with a tin cover. Under each cage a graduated cylinder was placed to receive the urine. After two hours from placing the animals in the cages the bladder was emptied by pulling the tail gently and the urine allowed to run down into the cylinder, then a reading was taken. The whole experiment was carried out under constant temperature (22°C. $\pm$ 1°) and in a quiet room. On the fifth day, after four days of complete rest, the experiment was repeated and the groups were crossed over so that each animal acted as its own control. To eliminate any source of emotional stress that may interfere with the diuresis, it was necessary first to train the animals to the conditions of the experiment for a week.

Results

As is shown in Table XIX a variable degree of water/

TABLE XIX.

Effect of Glycyrrhetinic Acid by Mouth on Diuresis in Rats.

Urine collection over two hours
(After a load of 12 ml H_2O)

No. of Rats	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Mean
Controls, ml urine	8	10	10	7.5	8	6.5	8.5	9	7.5	8.5	9.5	6	9	8	8.29
100 mg Glycyrrhetinic ml urine	3.5	4.75	3.5	6.5	6.5	6.5	5	1.5	7	4.75	7.25	4	7.25	5.5	5.25
$\frac{\text{Glyc.}}{\text{Control}} \times 100$	44	48	35	87	81	100	59	17	94	56	76	67	81	69	65

Each vertical column represents the values obtained from the same rat.

water retention was observed after the administration of 100 mg of glycyrrhetinic acid. The results after glycyrrhetinic acid were below the control values obtained from the same animals.

Propylene glycol, in doses of 1.0 ml by mouth caused in some instances, a mild haematuria. If the haematuria was severe, the result was discarded. The same animals showed less tendency to develop haematuria when they received glycyrrhetinic acid dissolved in propylene glycol. The response to the drug in animals developing haematuria did not show any difference from that obtained in non-affected rats.

The mean water retention in the experiments reported in Table XIX was 35%.

2. Diabetes Insipidus Rats.

Female albino rats (120-150 g were used. The posterior lobe of the pituitary gland was sucked out under ether anaesthesia, most of the anterior lobe being left intact. The operation was kindly performed by Dr. M. Vogt and Dr. M. Holzbauer. The rats/

rats were maintained on 5 per cent glucose drinking solution for two days with ordinary rat cubes for food. Penicillin treatment was administered as a protective measure for two days. After two days, each rat was put by itself in a metabolism cage and supplied with water and cubes ad lib. Urine was collected over 24 hours and compared with samples from intact rats of the same batch. Later, collection of urine was carried out over-night for 18 hours with water only supplied. Control intact animals were included in every experiment for comparative purposes. Over-night urine collections were continued during the glycyrrhetinic acid tests to measure the degree of diabetes insipidus present in every test rat during the whole procedure. This was necessary because in some of the operated rats only a transient phase of diabetes was achieved.

The test for the antidiuretic action of glycyrrhetinic acid was carried out only on those rats where the operation proved successful. When the diabetes subsided before the test was completed, the results were discarded. All the rats used in these/

these tests therefore, were diabetic throughout the test period.

The method used to test the effect of glycyrrhetinic acid on diuresis in the diabetes insipidus rats was the same as that employed for the non-anaesthetised rats. The same dose of 100 mg of the acid was introduced by stomach tube three hours before the 12 ml water load. A two hours' urine collection was carried out. Each rat acted alternately as control and as test animal. Two control values and two test values were obtained from each rat whenever possible. Before subjecting the rats to the operation they were trained for one week to get accustomed to the stomach tube and urine collection procedures.

Results.

The results of the operations were very variable, only in a few animals was the degree and duration of the diabetic phase satisfactory. In the last two rats reported here however, the operation was very successful, the maximal urine collection over-night/

over-night being 55 and 95 ml respectively. In Table XX, the degree of diuresis attained by the diabetic rats is presented. The values are calculated as percentage of urine secretion by non-operated controls of the same batch. The first values given are those for the sixth night after operation; they are followed by the figures for the nigh preceding and that following each glycyrrhetinic acid test.

TABLE XX

Diuresis after posterior lobectomy of the pituitary.

In rats

18 hr. collections, water ad lib.

No. of Rat	Urine secretion as % of control.				
	6th day after oper.	1st experiment[1]		2nd experiment[1]	
		Before	After	Before	After
1	190	93	311	-	-
2	141	224	239	376	372
3	190	93	710	-	-
4	270	195	533	106	286
5	1400	1830	350	370	330
6	2360	3170	510	650	400

[1]Before and after the corresponding glycyrrhetinic acid experiments -(Table XXI).

The figures in this table show that the degree

of/

of diabetes insipidus resulting from the operation was not steady from day to day. On the other hand, all showed a manifest increase in diuresis maintained till the animals died or were killed several weeks after the operation.

When glycyrrhetinic acid was administered to these rats, an inhibition of diuresis was observed, (Table XXI). This inhibition was comparable to that met with in non-diabetic rats. The mean water retention in these experiments was 41.5 as compared to 35 in the non-diabetic rats.

TABLE XXI.

Effect of glycyrrhetinic acid by the mouth on diuresis in diabetes insipidus rats.

Urine collection over two hours
(After a load of 12 ml H_2O)

No. of rat	Expt. No.	Control ml.	Glycy. ml.	$\frac{\text{Glycy.}}{\text{Control}} \times 100$
1	1	8.75	7.75	88.5
2	1	8.5	6.5	76.5
	2	7.0	6.25	89.0
3	1	10.5	6.0	57.0
4	1	3.0	1.5	50.0
	2	2.75	0.75	27.0
5	1	10.75	5.5	51.0
	2	9.5	6.0	63.0
6	1	10.0	5.5	55.0
	2	6.75	2.0	30.0

3./

3. Water absorption from the alimentary canal.

To see whether delayed water absorption from the gastro-intestinal tract played any part in the reduction of water excretion, the method used by Heller and Smirk (1932) was employed to investigate the effect of glycyrrhetinic acid on water absorption.

Adult albino rats of about 170 g weight were trained for one week to being fasted over-night and receiving a water load by stomach tube in the morning. The actual experiment consisted in fasting the rats for 24 hours allowing them free access to drinking water. The rats were then divided into pairs, selected to be identical in sex and weight. At 11.0 a.m. one ml of propylene glycol was administered by stomach tube to one rat of each pair and 100 mg of the acid partially dissolved in the same volume of propylene glycol to the other. The rats were kept in a quiet, warm place for three hours, at the end of which they received 5 per cent of the body weight of warm tap water by stomach tube. Fifteen to sixty minutes later the rats were killed and their intestinal tract dissected out and weighed. To

dissect/

dissect the intestinal tract, ligatures were tied around the cardiac end of the oesophagus and the anal end of the rectum, after removing any formed faeces. The canal was severed beyond the ligatures and dissected out, removing omentum and pancreas.

Before weighing, the caecum was opened and its contents emptied. Throughout the experiment, each pair of rats was handled together and kept in the same cage, conditions for the members of the same pair were therefore as identical as possible and the results obtained for the control and the glycyrrhetinic-treated rat comparable.

The dose of the acid, its mode of administration and the period elapsing until the administration of the water load were chosen so as to duplicate the procedures in the diuresis experiments reported before, in the hope that such an arrangement would facilitate the assessment of the results.

In another series of experiments, a 300 mg dose of the acid was given to the test animals, the contents of the caecum were not emptied before weighing the gut, and the liver was dissected out and weighed. The times allowed for water absorption were 15,/

15, 30 and 75 minutes.

Results.

In the first series of experiments (Table XXII) the weight of the gut, when expressed as percentage of body weight, was always higher in the test rat than in the control. This was the case whether the rats were killed 15 or 60 minutes after the water load.

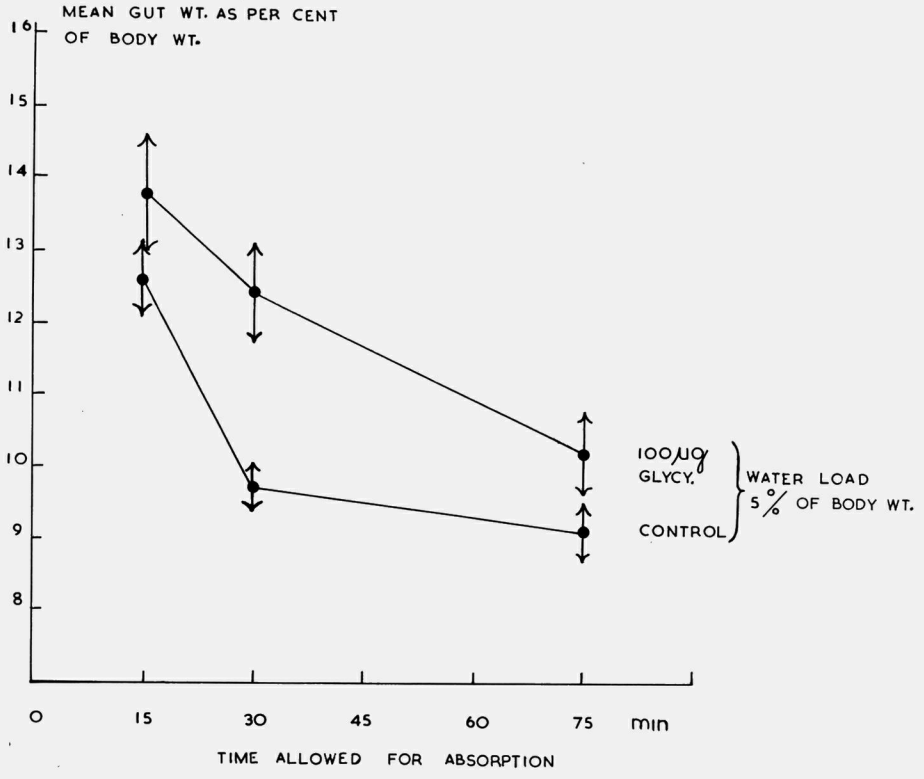
TABLE XXII

Effect of glycyrrhetinic acid by mouth on absorption of water from the gut of rats.

No. of Group	Weight of gut as % of body weight: CONTROL(a)	GLYCYRRHETINIC(b)	$\frac{b}{a} \times 100$ [1]
15 min after load			
1	8	8.9	111
2	8.6	9.6	112
3	9	9.4	105
4	7.4	8.6	116
5	8.3	10.	120
Mean	8.26	9.3	113
60 min after load			
1	6.2	7.2	117
2	6.6	8.5	129
3	5.4	5.8	107
4	6.2	7.5	121
5	6.2	6.4	103
Mean	6.12	7.08	115

[1]The differences between the mean of test and control rats are significant (P 0.01 - 0.001 after 15 min, P 0.02 - 0.01 after 60 min), provided the differences between each pair are used as the basis of the calculations.

Figure 18.



Effect of glycyrrhetinic acid on water absorption from the alimentary tract.

Each point represents mean for five rats, the arrows show the standard error.

(TABLE XXIII)

During dissection it was noticed that in the test animals, water appeared to be retained in the stomach, and no indication of the onset of diarrhoea was observed. In some of the diuresis experiments, a few rats developed diarrhoea after the same dose of glycyrrhetinic acid but the majority did not.

As is shown in Table XXII no significant difference in the degree of water retention was observed between the animals killed 15 minutes and those killed 60 minutes after the water load.

In the second series of experiments, three groups killed at different time intervals were employed. Each group consisted of ten rats, five receiving the acid and five acting as controls.

As is shown in Table XXIII and Fig.18 when the weight of the gut is calculated as a percentage of the body weight, the control groups give mean values lower than those of the test groups in the three periods. The maximal difference was observed in the 30 minute group. The range of the differences in the other periods was similar to that observed in the first series of experiments, where the dose/

dose of the acid given per rat was only 100 mg compared with 300 mg given in this second series.

The mean gut weight, in both control and test animals, decreased gradually with the length of time, indicating the gradual absorption of the intestinal contents. The controls show the same kind of curve as that described by Heller and Smirk (1932); a rapid rate of absorption in the first half hour after which the curve flattens out. In the test group, the same pattern is maintained with two differences; the first part of the curve is less steep and the whole curve is maintained at a higher level throughout and this is an indication of interferance with absorption.

The weights of the livers did not show any significant difference. Heller and Smirk found the liver to show the most marked response to hydration as shown by increase in weight; in this experiment however,no definite increase was found.

TABLE XXIII/

TABLE XXIII

Effect of Glycyrrhetinic acid on the Absorption of Water from the Gut and its Retention in the Liver.

Time since water load	No. of rats in group	Weight as % of body weight.			
		Control		Glycyrrhetinic (300mg)	
		Mean Gut Weight ±E	Mean Liver Weight ±E	Mean Gut Weight ±E	Mean Liver Weight ±E
15mins	10	12.6±.54	4.8±.41	13.8±.84	5.1±.25
30mins	10	9.7±.32	4.6±.29	12.4±.67	4.7±.25
75mins	10	9.1±.43	4.8±.16	10.2±.58	4.9±.1

When the rats received 100 mg of the acid (Table XXII), the mean weight of the gastro-intestinal tract was 6.12 per cent of body weight in the control group one hour after the water load and 7.08 per cent in the glycyrrhetinic acid group. This is a difference of 0.96 per cent of body weight or 19.2 per cent of the water load.

Heller and Smirk (1932) in their original experiments, showed that the weight of the gut drops in the first hour and reaches a level between 6 per cent/

cent and 7 per cent of the body weight at 60 minutes after the load, this level is maintained after that. They reported that absorption of a 5 per cent load is completed 15-20 minutes before the height of diuresis is reached. The 19.6 per cent difference between control and glycyrrhetinic acid groups can therefore, be taken as an estimation of the degree of water retention in the gut caused by 100 mg of the acid.

In the diuresis experiments reported in Table XIX, the differences between control values and those following 100 mg of the acid had a mean of 3.04 ml or 25.3 per cent of the load. The load however, in this case was almost double that used in the absorption experiments. The percentage of water retention in the gut is likely to be smaller with a bigger water load if the same dose of the acid is used. Therefore, it is unlikely that the delay in absorption of water on giving glycyrrhetinic acid by mouth accounts for the whole 25.3 per cent of water retained.

To investigate further the role played by gastro-intestinal/

intestinal water retention in the anti-diuretic action of glycyrrhetinic the following experiment was carried out.

Albino rats of 150 g weight were denied food for 24 hours. At 10 a.m. water was withdrawn and the animals divided into pairs according to weight. At 12 noon·2 ml of propylene glycol was injected intra-peritoneally into one rat of each group. The other had 10 mg of the acid dissolved in 0.2 ml of propylene glycol injected. A water load of 12 ml tap water was given by stomach tube ten minutes later. The rats were transferred to metabolic cages and a urine collection was carried out for 150 minutes. At the end of the collection, the rats were killed and their gastro-intestinal tract dissected out. The contents of the caecum were emptied out and the gut weighed (Table XXIV).

TABLE XXIV./

TABLE XXIV.

Effect of Glycyrrhetinic Acid Administered intra-peritoneally on Diuresis and Water Absorption.

(Water load of 12 ml)

Group No.	CONTROL		GLYCYRRHETINIC	
	Urine collection[1] % of load.	Gut weight % of body weight	Urine collection[1] % of load.	Gut wt. % of body wt.
1	70	7.7	20	7.8
2	30	9.3	2.3	7.9
3	30	7.3	5	8.4
Mean	43	8.1	9.1	8.03

[1]Urine collection carried out over 150 mins.

Results.

As shown in Table XXIV, 10 mg of the acid intra-peritoneally caused a marked degree of water retention as shown by the reduced degree of diuresis in/

in the test rats. The water retained in these rats is not retained in the gut as is evident by the identical weight of the guts in the control and test rats. In two of the test rats, however, a minor degree of peritoneal effusion was noticed which was not of a degree to account for the water retention.

DISCUSSION

a. Mineral metabolism.

The success reported by several workers (Groen et al. 1951, 1952; Card et al. 1953 and Pelser et al. 1953) in the treatment of adrenal deficiency with glycyrrhetinic acid and liquorice prompted the investigation of the mode of action of glycyrrhetinic acid on the salt metabolism of rats.

With the aid of the method of assaying mineral-cortical activity reported here, the following observations were made:

a) Under experimental conditions in which 1.0 µg DOCA caused a significant degree of sodium retention, 400 µg glycyrrhetinic acid caused no sodium retention, provided the collection of urine was/

was continued long enough to allow urinary flow.

b) A tendency to accelerate potassium excretion was observed, especially when a sizable potassium load was given.

The interpretation of these observations is not straightforward. As shown elsewhere, compounds E, F and B of Kendall when tested with this method of assay, did not retain sodium, on the contrary. sodium excretion was enhanced by all three. So while it may be justifiable to conclude that glycyrrhetinic acid does not behave like DOCA as far as the salt metabolism of adrenalectomised rats is concerned, it could not be overlooked that like certain cortical hormones, glycyrrhetinic acid, may still affect adrenocortical deficiency beneficially.

The experiments on the effect of the prolonged treatment of adult adrenalectomised rats recorded, however, confirm the impression that salt metabolism in these animals is not improved by glycyrrhetinic acid, while DOCA, as shown by the survival as well as by the significant changes in serum sodium, had a definite/

definite beneficial effect.

With a dose of 1 mg DOCA per day, the level of the serum sodium was significantly raised above the normal value. No significant drop in the serum potassium however, was noticed. Similarly, Buell and Turner (1941) reported almost complete restoration of the normal concentration of serum sodium while the serum potassium was only slightly reduced (see TABLE XXV).

TABLE XXV.

Serum electrolytes in adrenalectomised rats treated with DOCA.

Rat Serum	Normal	Adrenalectomised Non-treated	Adrenalectomised DOCA treated (pellet)
Sodium m eq per l.	144.2	133.7	141.4
Potassium m eq per l.	6.6	8.5	7.8

Glycyrrhetinic acid when administered in doses of/

of 3 mg per rat per day failed to produce any significant change either in the serum sodium or potassium. This dose is equivalent to 600 mg doses to a 60 kg man. Pelser et al. (1953) found 60 - 100 mg doses of the acid to be effective in Addisonian patients when given intramuscularly. Card et al. (1953) found 50 mg doses q.i.d. to be effective in a patient suffering from Addison's disease. The dose employed in the reported rats experiments falls well within the effective range in terms of these human doses.

These findings, therefore, confirm the findings of Nelemans-Stamperius (1949) and Card et al. (1953) according to which in rats, glycyrrhetinic acid did not cause significant changes in the electrolyte balance or survival times of adrenalectomised animals.

In Addison's disease, the degree of cortical deficiency is very variable, it is thus difficult to interpret the results of experiments carried out on such patients. The findings reported, in addition, are not consistent. While some workers found/

found the acid to have a DOCA-like effect, others found it to have no detectable beneficial effect.

Borst et al. (1953) treated two cases and found the drug effective only in one of them. In this last case, the more marked effect of the drug was its ability to accentuate the beneficial effect of small doses of cortisone. The authors thought the drug might be potentiated by the endogenous corticoids, and achieved some confirmation of their proposition by further experiments. They found the beneficial effect of the drug not to be reproducible in the previously responsive case while its potentiating effect on cortisone treatment was. In their hands, the drug was very effective in causing water and sodium retention, and so were combinations of liquorice with cortisone and with ACTH in a patient with Simmonds-Sheehan syndrome. The last combination, however, was not effective in an Addisonian patient, where one expects the endogenous corticoids to be either absent or severely reduced. Groen et al (1951, 1952) and Pelser et al (1953) on the other hand, found both liquorice and glycyrrhetinic acid to be effective in the treatment of Addison's/

Addison's disease.

Another aspect of the differences between liquorice and corticoid activity has been reported by Hudson et al (1953). Their study was carried out on two female post-menopausal and one male castrated patients all of whom have been totally adrenalectomised.

The amounts of 17-ketosteroids were estimated in 24-hour urine collections. The daily excretion parallelled the oral dosage level of cortisone acetate administered to the patients. Additional doses of glycyrrhizin in 4 g daily did not result in an increase in 17-ketosteroids excretion which became negligible when the patients received glycyrrhizin alone.

Our experiments indicate that glycyrrhetinic acid has no DOCA-like effect on the mineral metabolism of adrenalectomised rats. The reported favourable effects of the acid in Addisonian patients suggest two possible explanations: a) the drug may be metabolised in man in a different way giving by-products which are active in this respect or b) the mere water retention in Addisonian patients may have favourable effects under certain conditions.

b. Water metabolism.

Succus liquiritiae has been reported to cause water retention in man (Revers, 1948). This has been confirmed by Molhuysen et al. (1950); Groen et al. (1951) and Card et al. (1953).

Molhuysen et al. were able to produce their results with an extract of liquorice, mainly composed of glycyrrhizin. Groen et al. (1952) found that glycyrrhetinic acid, one of the components of glycyrrhizin, is the active principle.

On the other hand, water metabolism in laboratory animals did not show any definite response either to the succus or glycyrrhetinic acid; Nelemans-Stamperius (1949) and Card et al (1953).

In this series of experiments, it was possible to demonstrate the water retaining effect of glycyrrhetinic acid in albino rats and a dog.

In the rat, the acid has been introduced by mouth either as a fine suspension in 10 per cent ethyl alcohol or dissolved in propylene glycol; intravenously it was given dissolved in propylene glycol.

In/

In the dog, it was introduced by mouth as a coarse suspension in water or as a fine suspension in 10 per cent alcohol.

In all these cases, a variable degree of inhibition of diuresis was observed. The depression tended to be prolonged over a period of hours when the dose was big enough. Depression of diuresis did not show a uniform relation to dose. In a rat of 360 g, a dose of 40 µg in propylene glycol injected intravenously was found to be the minimum effective dose and in this case, the response was transient.

In all the previous reports, this effect of glycyrrhetinic acid has been related to its reported DOCA-like effect on mineral metabolism. The character of its anti-diuretic action in our experiments, seemed to differ substantially from the known DOCA-like effect on water metabolism.

Gaunt et al. (1949) pointed out that all the adrenal cortical hormones tested so far, have proved to/

to be diuretic. Gaunt (1943) and Gellhorn and Ballin (1946) showed that in intact animals all cortical hormones, including DOCA, promote water excretion and prevent water intoxication in cases of hydration.

Francois Morel (1951) reported a diuretic response to "Percorten" treatment in intact rats. Winter (1952) utilising DOCA in doses of 1 mg per day and cortisone in doses of 2 mg per day, confirmed the diuretic nature of both.

Kuhlman et al (1939) found that 25 mg of DOCA per day given to a dog increased its urinary output 2.5 times per day. Mushett et al. obtained similar results on dogs, (Mushett et al. 1951).

Winter and Ingram (1943) demonstrated that DOCA inhibits the tubular reabsorption of water. Boss et al. (1949) confirmed this observation and reported that very large doses also increase glomerular filtration.

The anti-diuretic effect of glycyrrhetinic acid in the reported experiments is obviously the opposite of the response following DOCA administration.

The/

The mechanism of the anti-diuretic action of this drug seems to be a complex one. No marked lowering of the blood pressure was observed when the drug was administered intravenously, which excludes the possibility of depressed renal plasma flow as a cause of anti-diuresis. The drug was effective in causing water retention in diabetes insipidus rats during the height of the diabetic phase. The rate of water retention in these diabetic rats was comparable to the rate of water retention in intact rats under the same conditions. The posterior lobe of the pituitary does not therefore, seem to play a role in this mechanism.

The drug, when given by mouth in doses identical with those used in the diuresis experiments caused a significant degree of water retention in the gut. The percentage of water retained in the diuresis experiments, however, was somewhat higher than the percentage of water retained in the gut. Intraperitoneally, the drug in small doses caused a marked suppression of diuresis and no water retention in the gut./

gut. Intravenously, the drug in minute doses when administered to alcohol anaesthetised rats, caused a marked depression of diuresis. In the sodium balance experiments, the drug was observed to cause water retention when administered subcutaneously.

By mouth, glycyrrhetinic acid seems to cause water retention in the alimentary canal as well as in the internal compartments of the body, possibly, through affecting the tubular reabsorption of water in the kidneys as suggested by Molhuysen et al. (1950). When given parenterally, only the second mechanism is involved.

The acid is precipitated in the stomach when it is introduced in propylene glycol and can still be detected there 4½ hours after administration, a possible explanation of the huge doses required by mouth.

When urine secretion was completely suppressed, temporary retention of sodium was the consequence, a feature worth investigation in balance experiments on Addison patients treated with glycyrrhetinic acid.

SUMMARY.

Glycyrrhetinic acid has been used to investigate the reported DOCA-like activity of liquorice extracts and some of its components. When given to adrenalectomised rats no effect on the sodium balance was observed. Only during the period in which diuresis was suppressed was there a retention of sodium.

The effect on the potassium balance was not significant unless the level of the extracellular potassium was highly elevated.

Prolonged treatment of adrenalectomised rats caused no significant improvement in the level of serum electrolytes, weight or survival.

Given by any route, the acid had an antidiuretic effect. The posterior lobe of the pituitary is not involved in this action of the drug, which may be on the tubular reabsorption of water.

When/

When given by mouth, the drug delays water absorption from the alimentary tract. The delay is sufficient to contribute to the over-all water retention effect of the drug when administered orally.

It is concluded that the actions of the drug follow a pattern different from that followed by DOCA.

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